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EFFECT OF PROTEIN SOURCE AND AMINO ACID SUPPLEMENTATION ON INTESTINAL MICROFLORA AND PLASMA AMINO ACIDS OF THE CHICK

by

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INTRODUCTION

A protein molecule is made up of a great number of amino acids residues combined in a particular manner with each other. Different proteins vary in their amino acid content and also in the availability of these individual amino acids to the chick and to other species. Nutritionists, therefore, must be aware of amino acid deficiencies and differences in availabilities to be able to balance a ration for adequate growth. One serious amino acid deficiency is enough to cause the failure of the entire ration.

There are, of course, cases in which it can be demonstrated that a change in the proportion of an amino acid in the diet, quite apart from a deficiency, can cause adverse effects on chick growth or on some other physiological process. The detrimental effects that result from the ingestion of excessive amounts can be attributed to amino acid toxicities.

The concept of amino acid balance is founded on our knowledge of the relationship between the amino acid composition of a protein and its biological value. A balanced protein, or one with a high biological value, provides amino acids approximately in proportion to the chick's, or other species, requirement. A poor protein, or one which is low in several essential amino acids, is an unbalanced protein and

and has a low biological value.

The unsatisfactory rate of growth of chicks consuming an unbalanced protein is frequently attributed to an amino acid imbalance. These imbalances are probably also influenced by other factors and, apart from the fact that it is necessary to provide amino acids in certain definite proportions in order to meet the chick's requirements, our knowledge of the limits within which the amino acids can be altered is quite sparse.

The nutritional value of a protein for the chicken is influenced by the extent to which the amino acid content of the protein agrees with the chick's essential requirements. A variety of protein sources in the diet is likely to be more complete and balanced in amino acids than is a single protein source.

These experiments were designed to obtain some insight into the interrelationship of two critical amino acids, arginine and lysine, in the chick's diet on the intestinal microflora and the free plasma amino acids of the chick.

REVIEW OF LITERATURE

Arginine Requirement

In 1936 Arnold et al. reported that a chick diet containing casein as the main source of protein could be improved by the addition of arginine. At the time this was a particularly striking find to the authors, since casein contained 4.8 percent arginine.

Weitlake et al. (1954) determined that the arginine requirement of the chick fed a casein diet was 1.7 percent of the total diet. This was notably higher than the 1.2 percent arginine that Almquist (1952) had found necessary on a practical diet.

Fisher et al. (1956) reported that during the first three weeks of life of the chick, the arginine requirement for growth and feed efficiency is greater than 1.3 percent and possibly as high as 1.9 percent arginine on a 25 percent casein diet.

A purified diet with 35 percent casein as the sole protein source was found to be grossly deficient in arginine as determined by Hogan et al. (1957). The deficiency was almost completely remedied by adding 1.2 percent arginine to the diet. The optimum amount of total arginine was over 1.8 percent but less than 2.5 percent.

Fisher and Johnson (1957) obtained similar growth in a seven day trial with a free amino acid diet as with a practical diet. Their purified diet contained 2.0 percent L-arginine HCl and also a very high concentration of lysine, as 1.6 percent L-lysine HCl (95 percent). The ability of the chick to synthesize many non-essential amino acids was demonstrated by the fact that their diet contained only L-tyrosine as a non-essential amino acid.

Snyder et al. (1954) noted considerable variation between chicks with respect to the requirement for arginine, but also showed that growth increased with increasing levels of arginine, up to 1.68 percent, on a 22 percent casein diet. In another experiment, the authors found it necessary to substitute 12.24 percent gelatin on an 18 percent casein diet to obtain optimum growth. This was equivalent to 1.67 percent arginine.

In later work Snyder et al. (1956) demonstrated that gelatin supplementation of a practical corn-soya diet, containing 1.11 percent arginine, did not improve growth, but that 1.73 percent arginine was necessary for maximum growth on a 22 percent casein diet. The authors stated that the presence of creatine in natural feed ingredients probably explains the lower requirements for arginine on practical diets.

Earlier, from the same laboratory, Griminger et al. (1955) reported that the arginine requirement for the chick appeared to be higher than 1.25 percent of the total diet. The authors felt that the greater chick gains obtained on the higher arginine levels were accentuated by the chick's high requirement for this amino acid for muscle creatine synthesis.

Krautmann et al. (1957) reported that the arginine requirement on a 21 percent dextrose-casein diet was 1.65 percent, but when gelatin was added as a source of arginine, 1.4 percent arginine was adequate. When a corn-casein diet containing 1.25 percent arginine was fed, growth obtained was comparable with that obtained from a casein diet containing 1.66 percent arginine. It was concluded by the authors that there was an indication of an unidentified factor of plant origin which enhanced the utilization of the amino acid or otherwise rendered the arginine in casein and practical feeds more available to the young chick.

More recently Krautmann et al. (1958) reported that corn and soybean oilmeal contained a factor which lowered the level of arginine required by the young chick. The authors stated that the growth response appeared to be more vitamin-like than a protein or amino acid response, because small changes in amino acids do not produce increased weight gains of the magnitude they obtained on their experiments.

Using surgically altered birds to make possible separate collection of urine and feces, O'Dell *et al.* (1958) studied the nitrogeneous excretory products in urine and feces of 5-week-old chicks fed different rations of casein, casein-gelatin or casein with added arginine. The authors noted that urinary arginine accounted for about 1 percent of the arginine consumed and did not vary with the diet. Their studies also showed that only 2 to 5 percent of the arginine consumed was in the feces with little or no difference between diets. Creatine and creatinine excretion was also quite constant from one diet to another. The daily excretion of urea varied with diets and increased as the intake of arginine increased. Urea excretion accounted for about 30 percent of the arginine consumed in the case of the practical diet and for more than 40 percent when one-half of the arginine was supplied as the free amino acid.

The authors postulated that the higher requirement for arginine on casein diets was due to the more rapid absorption of the free amino acid and consequently a more rapid breakdown to urea by kidney arginase.

Scott and Forbes (1958) reported that the addition of cellulose, at the expense of carbohydrate, to a casein-cerelose purified diet severely deficient in arginine, in

creased feed intake and improved chick growth. If, however, the basal diet was supplemented with increasing increments of L-arginine-HCl, they reported that the corrective action of cellulose became progressively less and disappeared entirely at the highest level of arginine supplementation. Additions of cellulose at the expense of the basal mixture did not improve growth on the arginine deficient diet because consumption of effective nutrients was not increased. Similarly, cellulose supplementation did not alleviate the deficiency of arginine on iso-caloric diets. The authors concluded that the improvement reflected no more than the narrowing of the calorie-protein ratio.

Anderson and Dobson (1958) showed that the arginine requirement on a casein diet supplemented with amino acids was 1.4 percent. Using combinations of Drackett protein, zein, and supplementary amino acids, the arginine requirement was determined to be 1.0 percent. The authors concluded that the difference in the amino acid composition of the two diets appeared to be the main factor responsible for the differing arginine requirement.

In attempting to duplicate the results obtained on casein diets, Klain et al. (1959) used a crystalline amino acid mixture that was similar to the amino acid composition of casein at a 30 percent protein level and determined that

the arginine requirement of the growing chick was 2.06 percent. This would indicate that the amino acid make-up of casein was involved in the high arginine requirement of chicks on this diet.

Fluckiger and Anderson (1959) investigated hormonal action on arginine by adding thiouracil to casein diets deficient in arginine. They obtained an increased growth rate while at the same time lessening the severity of the arginine deficiency symptoms. They also noted that the amount of arginine required for maximum growth was decreased by thiouracil. Additions of thyroxine or iodinated casein produced the opposite effect.

Fisher et al. (1959) reported that neither the growing or mature chicken can synthesize arginine in amounts large enough to permit normal growth or maintenance to occur. The authors also noted that neither glutamic acid or proline exerted a sparing effect on the arginine needs of the growing or mature chicken.

More recently, Fisher and Griminger (1960) demonstrated that the performance of laying hens on low protein diets, which were also low in arginine, could be improved by the addition of gelatin. The criteria used were improved body weight, egg production, and egg size.

Lysine Requirement

Using a 20 percent protein diet for both chicks and poults, Grau et al. (1946) determined the lysine requirement for chicks to be 0.9 percent and for turkeys 1.3 percent. It was their conclusion that the poult requirement is higher than the chick's by an amount roughly proportional to the higher protein requirement of poults.

In 1956 Edwards et al. reported the chick's lysine requirement appeared related to rate of growth. Slower growing chicks fed a wheat gluten meal diet required 0.9 percent lysine for good growth.

Hill (1953) used sesame meal as a protein source and concluded that 0.9 percent lysine was a conservative estimate of the chick's lysine requirement which appeared to be dependent on protein level and rate of growth. He felt that 1.0 percent lysine was a better estimate of the actual requirement and that the difference or higher requirement was in part due to the destruction of protein quality, particularly of lysine content, in processing of different meals.

At about the same time, Balloun et al. (1953) demonstrated that the growth-inhibiting effects of raw soybean oil mealwere progressively inactivated by autoclaving. Substantially complete inactivation was obtained when the meal was

autoclaved 20 minutes at 15 pounds of steam pressure as determined by growth response of young chicks. Heating soybean oil meal for periods longer than 20 minutes reduced the lysine activity and the nutritive value of the meal.

Vohra and Kratzer (1957) reported that single daily supplementation of lysine was inferior for growth and feather pigmentation as compared to the same amount of lysine present in the feed where other amino acids are also present. The authors concluded that this strongly suggested a "protein-function" of lysine. This was further confirmed by the fact that their experiments did not separate the function of lysine for the formation of pigment from its requirement for growth. The authors stated that if lysine acts as a precursor of some vitamin which is involved in the development of pigment, a single supplement of it should have improved the color of the feathers more than the growth of the birds. If lysine is a precursor of a polypeptide hormone, it could play an important role both for growth and for feather pigmentation.

Klain et al. (1957) demonstrated inhibition of pigment deposition in the feathers of four different breeds of chickens fed a lysine deficient diet based on sunflower seed oil meal. The achromatosis was particularly evident in the primary wing feathers and the males seemed to be more severely affected than did the females. Several substances known to

be concerned with pigmentation were added such as copper, folic acid, iodinated casein, L-tyrosine and lysine. Tyrosine reduced the severity and lysine supplementation prevented the achromatosis. The structure of the feather protein was not altered by the lysine-deficient diet.

More recently, Klain et al. (1960) developed a crystalline amino acid diet on which they reported no depigmentation and obtained very good growth, 9 grams per chick per day, during the second week of life. The lysine level was 1.01 percent of the diet and the arginine level was 1.08 percent.

Schwartz et al. (1959) studied the utilization of protein-bound lysine and free dietary lysine and found no difference. The authors used growth improvement as a measure of response.

Amino Acid Imbalance

Either an excessive or deficient supply of any one of the essential amino acids in relation to the supply of the other amino acids has been shown to be detrimental to the growth of poultry, swine and rats. Over fifty years ago, Willcock and Hopkins (1906) recognized the importance of the proportions of individual amino acids in dietary proteins. They fed mice a diet composed of zein as the only nitrogen source. They noted that this diet did not maintain the growth of young mice, and the additions of tryptophan to the diet did

not improve growth rate, but did improve the survival time of the mice. Tyrosine had no such effects because, as they noted, it was present in zein in adequate amounts.

In the early work of Osborne and Mendel (1914) it was reported by the authors that it was as logical to assume that the maintenance protein requirement was in reality a requirement for definite amino acids that serve special physiological functions as it was to assume that protein, as such, was needed to repair a hypothetical destruction of the entire protein molecule.

In his excellent review on amino acid balances and imbalances, Harper (1958) noted that the severity of an amino acid deficiency could be increased by providing in the diet a supplementary amount of an amino acid or a mixture of amino acids other than the one which limited growth. It should be noted that similar methods are sometimes used to determine amino acid requirements in which protein, or amino acids that make up the protein, are increased and the amino acid which is being studied is then added in increasing increments. He also noted that the creation of amino acid imbalances or toxicities may some day find a therapeutic use.

Work which lends evidence to Harper's theory was reported by Gershoff et al. (1952) in which mice exhibited decreased susceptibility and prolonged survival time to Lansing

poliomyelitis, when fed supplemental amino acids. The addition of 5 percent DL-methionine or 0.4 percent 6-methyl tryptophan to a 9 percent casein diet would accomplish this. The addition of both in combination resulted in even more protection.

More recently, Harper (1959) proposed a definition of an amino acid imbalance as:

> Any change in the proportions of the amino acids in a diet that result in an adverse effect which can be prevented by supplementing the diet with a relatively small amount of the most limiting amino acid or acids.

Similarly, Woolley (1952) discussed the antagonistic properties of amino acids. This antagonism involved amino acids that are very similar in structure in which one will interfere with the metabolism of another amino acid.

Woolley (1952), stated in his book on antimetabolites that individual members of a pair of antagonistic amino acids are usually quite similar in structure, but they are even more specific in their action. As an example, Doermann (1944) discovered a mutant strain of Neurospora which required lysine as a nutrient. In contrast to most higher animals, wild strains of Neurospora do not require amino acids as nutrients. They will usually grow in an aqueous medium to which only inorganic salts, a carbon source and biotin are added. When

arginine was added to the medium also, at a molecular ratio of about one arginine to one lysine, the growth of the mutant was reduced to one-half as compared to that in arginine-free medium. If the ratio of arginine to lysine was doubled, growth was completely inhibited. Of other amino acids tried, only tryptophan and norleucine inhibited growth, and these only slightly.

A growth depression was observed by Henderson et al. (1947) in rats receiving a 9.0 percent casein-sucrose, niacin-free diet upon supplementation with 2.0 percent glycine, 2.0 percent acid-hydrolyzed casein or the crystalline amino acids in amounts contained in 2.0 percent acid-hydrolyzed casein. The growth-depressing effect of glycine was overcome by substituting dextran for sucrose in the diet.

Stevens and Bush (1950) obtained a growth response in rats when homoarginine was added to a lysine-deficient diet. The growth response was delayed and quite small, but it was consistently observed in all the experimental animals.

Hardin and Hove (1951) demonstrated that the toxic effects of excessive methionine in the ration of rats could be partially overcome by the addition of molecular equivalent quantities of glycine and arginine. They theorized that since glycine and arginine are precursors of creatine, the detoxi-

fication mechanism was accomplished by the using up of excess methyl groups from methionine to form creatine.

Ebisuzaki et al. (1952) fed a similar casein-sucrose, niacin-free diet to rats, as well as a diet containing synthetic amino acids in amounts similar to those in casein. Added DL-threonine was growth-depressing in the casein diet, but not in the synthetic amino acid diet. The authors concluded that the excess dietary threonine decreased the availability of amino acids in casein by inhibiting digestive processes. They also injected threonine and obtained similar results. The injected threonine, they postulated, may have been returned to the intestinal lumen via the bile.

In discussing amino acid imbalances in a ration, it is pertinent that one consider the effects of some imbalances upon the rate of absorption of amino acids from the intestine. Pinsky and Geiger (1952) reported that the absorption of proper concentrations of essential amino acids was in many cases hindered by the presence of excessive amounts of other amino acids. They concluded that an imbalance of amino acids might result from the differential absorption of amino acids from the gastrointestinal tract.

Using a casein-starch diet, Russell et al. (1952) added each of the essential amino acids to the basal diet at a

level 200 percent above the suggested requirement of the rat. They found growth depression with only DL-lysine, DL-methionine and DL-valine. These authors suggested that excess amino acids *per se* are not toxic to animals, but that the degree of toxicity is relative to a particular amino acid and to the nutritive makeup of the diet.

Munaver and Harper (1959) observed that the effects of wheat gluten diets resembled the effects of an amino acid imbalance, since it was necessary to add lysine to increasing levels of wheat gluten to prevent a fall in growth rate. When the level of wheat gluten was at 60 percent to meet the rat's requirement for lysine, all the other indispensable amino acids in the diet were much above their requirements. The authors conclude that wheat gluten, due to its very low level of lysine, is so severely unbalanced that the lysine it contains is not completely utilized.

Similar amino acid imbalances have been noted in the chick. Sanders *et al.* (1950) noted that the addition of lysine alone to a chick diet deficient in lysine, arginine and methionine caused an abnormal feathering which was characterized by curved and degenerate feathers. When arginine was added along with lysine, the feathering of the birds was normal. The diet was based on milo-gluten meal and contained 22 percent protein. The authors felt that the addition of

lysine without arginine apparently created a severe deficiency or imbalance of arginine, resulting in the abnormal feathers. They did not note any feather structure abnormalities in the basal group. They did, however, note a slight depigmentation which they concluded was due to a lysine deficiency.

Anderson and Combs (1952) noted that the growth depression obtained by the feeding of high levels of DL-lysine-HCl was proportionately less when zein, corn or additional gelatin was added to the diet. The growth depression caused by excessive tyrosine was also less when additional gelatin was added. These proteins increase the level of other amino acids in the diet and thus appear to provide a more favorable amino acid balance. Even though a better dietary amino acid balance was obtained by the addition of arginine and gelatin, the authors could not decrease the growth depression caused by the feeding of excessive methionine. They concluded that the restricted feed intake was largely responsible for the growth depression.

Using broiler chicks, Donovan et al. (1959) observed that when the lysine level was 1.2 percent optimum growth could be obtained with a methionine level between 0.46-0.76 percent. When methionine was constant at 0.46 percent, optimum growth was obtained at total lysine levels ranging from 1.2-1.4 percent. The authors also noted that the lysine

requirement increased from 1.02-1.12 percent as the productive calories increased from 923-1,018 per pound of diet.

In earlier work at this laboratory, Owings and Balloun (1959) noted that with a casein-zein diet containing a low lysine level and a low arginine level, birds exhibited depigmented areas in their feathers. This condition was completely corrected by increasing the lysine content of the diet. They also found that if the lysine was kept low and the arginine level increased to 1.7 percent only moderate or no depigmentation occurred. The increased arginine also increased the feather-tyrosinase activity, although not as much as did lysine supplementation. The authors concluded that arginine may also be involved in some manner in melanin formation.

Klain et al. (1960) stated that achromatosis in chicks occurs when there is a low intake of lysine accompanied by an excess intake of other amino acids and is not caused by a low intake of lysine per se.

Anderson and Dobson (1959) conducted experiments which indicate that the amino acid composition of casein is responsible for the higher arginine requirement when casein provides most of the protein in the chick diet. The same high arginine requirement was also noted with diets based on other proteins with amino acids added to provide the same essential

amino acid content as a ration based on casein. The authors concluded that the increased arginine requirement is based on amounts of other amino acids in the diet, since ration protein *per se* had little effect on the requirements for arginine and lysine.

Calvert *et al.* (1960) showed that in chicks fed a purified diet containing casein as the principle source of protein and deficient in arginine, sulfur amino acids and vitamin E, no muscular dystrophy occurred up to 7 weeks of age. When the diet was supplemented with 1, 2 or 4 percent arginine-HCl, severe muscular dystrophy occurred in 90 percent of the chicks by the time they were 5 weeks of age. If the basal diet plus arginine was supplemented with either methionine, cystine, or vitamin E, muscular dystrophy was prevented. The authors concluded that their results indicated the existence of a very important interrelationship among the sulfur amino acids, arginine and vitamin E.

Free Amino Acids of Blood Plasma

Hier and Bergeim (1946) determined certain free plasma amino acids microbiologically in the dog and human and found the average amounts of arginine, histidine, lysine, phenylalanine, tyrosine and tryptophan to be substantially the same in the two species.

In later work, Hier (1947) determined that when arginine was fed to dogs at the rate of 0.56 gm per kilo of body weight, arginine in the blood plasma increased 17 times. There was no change noted in the other amino acids, with the exception of tyrosine, which decreased slightly and lysine, which showed a small increase.

The author also studied the excretion levels of amino acids when supplemental leucine was fed. They found no increased excretion of any amino acid except leucine. The decreased concentration of several other free amino acids in the plasma, when free leucine was fed, could not be explained on the basis of increased excretion. The author postulated that the same amino acid oxidase or deaminase may be involved and that when large amounts of leucine were metabolized, the other amino acids were also removed from the plasma.

In measuring 18 free plasma amino acids of the pig fed a practical diet, Richardson et al. (1958) found animals within a treatment quite variable. In comparing different age groups, the authors found that plasma glutamic acid and valine were significantly higher in the 40 pound pig and methionine, lysine and leucine were significantly higher in 100 pound pigs. Age and weight had no effect on blood concentrations of the 13 other amino acids.

Frame (1958) studied the free amino acids of plasma in humans fed a high protein diet and found that most of the amino acids increased in concentration after such a meal, but the rise did not parallel the relative amino acid composition of the food; nor did the relative concentrations of the different amino acids in the plasma remain constant.

Longenecker and Hause (1958) used dogs to compare the rate of absorption of a free amino acid supplement with that of amino acids liberated by the digestion of a test protein. They found that the rate of absorption for the free amino acids was similar to that of amino acids of the test protein. In later experiments, Longenecker and Hause (1959) found the plasma amino acid changes of the adult dog after a meal to be directly dependent upon the amino acid composition of the protein ingested.

In studies concerned with the free amino acids of blood plasma of chicks, Richardson et al. (1953a) demonstrated that when the diet was low in pyridoxine or pantothenic acid, the blood concentration of lysine was higher than when the diet was adequate in these vitamins. Blood arginine and lysine were not affected significantly by low dietary riboflavin levels.

Richardson et al. (1953b) showed that the concentrations of free amino acids in the plasma were directly correlated to

the concentrations of the same amino acids in the diet. They also showed that the addition of L-lysine to the diet increased the concentration of free arginine, lysine and valine in the blood plasma.

Using paper chromatography procedures, Ross *et al.* (1955a) found that arginine, methionine, glycine and tryptophan were at lower concentrations in the plasma of birds infected with *S. pullorum*. The authors conceived that the organisms may utilize those amino acids while parasitizing the host. However, previous *in vitro* studies have not revealed a need for the four amino acids by *S. pullorum*.

More evidence for Harper's (1958) theory of the possible therapeutic value of amino acid supplementation was reported by Ross *et al.* (1955b). Free arginine, when fed to birds infected with *S. pullorum*, increased their survival time. The authors did not note any stimulation of phagocytic activity, and arginine did not show any toxicity for *S. pullorum* in *in vitro* studies.

Chubb (1959) studied the amino acid patterns of blood plasma of young chicks using paper chromatography and found that the blood plasma amino acids of various age groups showed remarkable consistency of size and color intensity upon development. Age and sex were found to have no effect on amino acids of the blood plasma. The dominant amino acids noted

were alanine, glutamic acid, glycine, leucine, lysine, valine, serine and threonine. The weakest spots were hydroxyproline, cystine, cysteic acid, methionine, tyrosine, and tryptophan. Arginine, aspartic acid, proline, phenylalanine and taurine gave moderate color.

Chicks fasted for 18 hours gave a different pattern of amino acid concentration than nonfasted birds. The nonfasted birds showed less alanine, glutamic acid, glycine, serine and threonine, but a greater concentration in prolines, cystine and cysteic acid when compared to fasted birds.

The author also noted that urinary amino acid patterns of the fowl were found to be very similar to that of their blood plasma.

Olsen et al. (1959) studied 12 amino acids in protein-free plasma of chicks fed purified diets ranging from 8.5 to 30 percent protein. Glycine, isoleucine, leucine, lysine, methionine, threonine and valine exhibited a distinct rise in concentration in the plasma as the protein content of the diet was increased. The other group of amino acids, consisting of arginine, histidine, phenylalanine, tryptophan and tyrosine, either rose only slightly, maintained a relatively constant concentration, or decreased. The authors suggested that amino acids containing a ring structure or a double bond

in the molecule respond more readily to mechanisms operating to maintain or reduce their plasma levels.

O'Dell et al. (1960) detected hydroxyproline in chick urine, which they noted had not been reported in mammalian urine. In their studies, glycine accounted for 20 percent of the urinary amino acid nitrogen. Proline, hydroxyproline, arginine, ornithine, lysine and glutamic acid made up the majority of the remainder.

Intestinal Microflora

Despite numerous studies conducted to determine the role of intestinal bacteria, their functions are not completely understood. The kind of bacteria found in the digestive tract and their distribution have been studied in many animal species including the fowl.

The relationship of intestinal bacteria to the well-being of the fowl has been of research interest for over seventy-five years, dating back to the observations by Pasteur (1885), that microflora in the intestinal tract of the chicken was necessary for its existence. One of the first studies of the microflora of the chicken's intestinal tract was conducted by Kern (1897) and one of the first extensive germ-free studies in chicken was reported by Cohendy (1912).

In 1905, King determined that the intestinal flora of

the chicken depends somewhat upon environment. He also reported a greater abundance of organisms in the ceca and colon than in the other portions of the intestine.

Gage (1911) determined that there was a greater number of organism in the duodenum in contrast to the findings of King (1905), and also that there were very few obligatory anaerobes present. He reported too that the intestinal flora of healthy birds varies with environment.

Emmel (1930) agreed with the work of Gage (1911) in the determination of the presence of few obligate anaerobes. He also reported the coliforms to be the predominate organisms found in the feces of two-week-old chicks and older hens. These averaged 60 percent of the total organisms found.

Porter and Rettger (1940) reported that the bacterial flora of the rat could be altered by the diet, but was generally quite stable. This view was also confirmed in the chicken by Johansson et al. (1948). They found that the type of carbohydrate influenced the microflora of the fecal droppings of hens. Dextrin was found to stimulate the development of considerable numbers of coliforms. Lactose also increased coliforms, but lactic acid bacteria were more numerous. In general, they found that intestinal microflora increased in numbers from the duodenum to the ceca, in contrast to the report of Gage (1911). Yeast and enterococci were found to in-

crease the least, and birds on dextrin had the highest microbial counts.

In studying cecal feces from turkeys, Harrison and Hansen (1950) found anaerobic lactobacilli as the most numerous bacteria, making up about 50 percent of the total cultivable flora. Micrococci were found to be the most variable with some feces containing large numbers, whereas they were never recovered in many others. In ceca flora, anaerobic bacteria were in the majority, and almost the entire flora was composed of gram-positive organisms. Coliforms and enterococci were noted to exist in relatively small numbers, each comprising less than 1 percent of the flora. The authors postulated that earlier reports of the predominance of coliforms in the feces of poultry was perhaps due to the ease of isolation and identification as compared to other organisms.

There is extensive literature on the effects of antibiotic feeding on the intestinal flora of poultry and other species. It is not within the scope of this review, however, to include all of this literature, but rather to confine it primarily to the effects of various nutrients and other substances.

One aspect of antibiotic feeding which may apply is the feeding of organisms obtained from birds on antibiotic supplementation to young chicks. Anderson et al. (1950) reported

that feeding coliform cultures to poults resulted in some improved weight gains. No consistent alterations were noted in ceca flora by coliform feeding.

Later, from the same laboratory, Anderson, Slinger, and Pepper (1952) found that the feeding of typical and atypical E. coli to chicks in the presence of penicillin resulted in increased weight gains. In the absence of penicillin, micrococci decreased growth of chicks.

Anderson et al. (1953) fed anaerobe cultures to chicks and noticed no increase in weight, but did note improved feed efficiency.

More recently, Bogdonoff et al. (1959) demonstrated that coliform inoculations per se appeared to be without effect on growth or feed efficiency. They did, however, obtain growth responses from combinations of antibiotics plus coliforms.

Anderson, Cunningham, and Slinger (1952) noted also that in the absence of penicillin, increasing the protein level from 17 to 26 percent failed to alter the counts materially. Anaerobes and microaerophiles tended to increase some as the protein was increased to 23 percent. Also, the proteolytic count was highest at the 26 percent protein level.

The feeding of B-grade molasses to pullets was reported by Wiseman *et al.* (1956) to reduce the intestinal total aerobe and lactobacilli counts. The authors noted an excessively high water intake by birds on the molasses diet which they postulated might have decreased the microflora counts by dilution or by increasing peristalsis. The more rapid digestion of the B-grade molasses, as compared to yellow corn, could have also altered bacterial growth.

Anderson *et al.* (1957) demonstrated that in the absence of Aureomycin, increasing levels of manganese and/or niacin tended to increase the numbers of all types of cecal organisms with the exception of the enterococci. Aureomycin increased counts on all diets except the one containing 50 ppm manganese and 27 mg niacin per pound.

In experiments in which bacitracin and penicillin were added to a folic acid-deficient grain ration, Wiseman (1958) reported that replacing 50 percent of the grain with sucrose produced more beneficial alterations in the chicken's intestinal microflora than did other modifications to the diet. The more beneficial effects were expressed in a decrease of folic acid requiring lactobacilli and an increase in the folic acid-synthesizing *Aerobacter* species. Although the antibiotics mentioned would decrease the lactobacilli counts, the added sucrose would increase the synthesizing organism as

well as decrease the lactobacilli which utilized the folic acid.

With the evidence presented that environment and ordinary dietary feedstuffs can alter the microflora of the chicken, it is of interest to note the development of this flora.

In experiments undertaken by Lev and Briggs (1956a) it was demonstrated that the ceca of newly hatched chicks contained a dense flora including many clostridial spores; whereas, the other organs were sterile or contained only very few organisms.

Lev and Briggs (1956b) reported that the development of the bacterial flora in the gut of the chick takes place within 24 hours after the first feeding. They also made counts from six alimentary organs from groups of birds of increasing ages. They noted that total counts from the crop and ceca were relatively stable, while those of the intestine fluctuated a great deal.

It is of interest to note that the intestines of antarctic birds are much lower in intestinal flora when compared to domestic fowl, but their digestive tract is not sterile as had once been proposed. Sieburth (1959) and also McBee (1960) were not able to isolate any aerobic microorganisms

from the intestine of antarctic birds, but were able to cultivate anaerobic bacteria. This may have accounted for the earlier reports of sterility. Another factor both authors considered possible was the antibiotic effect of the algae found in the digestive tract of shrimp, which make up a large part of the diet of these birds.

EXPERIMENTAL PROCEDURE

Stock Used

Vent-sexed Vantress x Arbor Acre male chicks obtained from a commercial hatchery were used in all experiments.

Methods of Feeding and Management

The experiments were conducted in five-deck battery brooders equipped with wire floors and thermostatically controlled electric heating elements of the back-warming type. The temperature under the hovers was adjusted to the comfort of the chicks from an initial temperature of 100°F. The hovers were stationary at a height of four inches throughout the experimental period. All experiments were carried out in a room where the temperature was maintained at approximately 70°F.

The experimental rations and water were provided ad libitum. At the start of the experiment, sufficient feed for the test period was mixed for each experimental lot and stored in metal cans with covers.

Records and Experimental Data

The male chicks were wing-banded and weighed as a group at one day of age and immediately placed on experimental rations. All experiments were of 4-week durations, except

Experiment V which was 2 weeks.

Chicks were weighed bi-weekly as a group in an experimental lot. Mortality records were kept daily and feed consumption data tabulated.

The analyses of variance of all experimental chick data were made according to the method described by Snedecor (1956).

Microbiological and Chemical Tests

Microbiological procedure

A plate count technique with selective media was used for all organisms studied. This technique makes two major assumptions and is subject to the limitation thereof: (a) each colony develops from only one cell and (b) all organisms than the ones desired fail to multiply at a rate fast enough to produce visible colonial growth in the allotted incubation time.

One gram samples were weighed into sterile aluminum dishes and initially diluted, by weight, one part sample to ninety-nine parts sterile, buffered water. This mixture was then blended for two minutes in a semi-micro Waring blendor with serial decimal dilutions made in sterile buffered water, each dilution being mixed by shaking 25 times before each transfer. The buffered dilution water was prepared by adding

34 grams of KH_2PO_4 to 500 milliliters 1M NaOH and making the volume up to 1 liter. Then 1.25 milliliters of this stock buffer solution were added to 1 liter of distilled water for the final solution.

All plates were inoculated with 1 milliliter of the dilution and only one plate per dilution was used. Wilbur (1959) showed that this system provided enough different dilutions so that 1 milliliter portions could be used for inoculation of all plates in the dilution series. This system provided more consistent results and eliminated the need for replicate plates. As each plate was inoculated, exactly 18 milliliters of the desired medium were added by use of an automatic pipetting machine. It was then mixed with the inoculum by moving the plate in an epicycloid motion, after which the agar was allowed to solidify.

The group of organisms studied and the selective media used were as follows: (a) coliform - violet red bile agar, (b) lactobacilli - tomato juice agar, special, (c) total aerobes - tryptone glucose extract agar, (d) total anaerobes - fluid thioglycollate medium, plus 1.5 percent agar, (e) streptococci - mitis salivarius agar plus 1 milliliter of one percent solution of potassium tellurite per liter, (f) staphylococci - staphylococcus medium No. 110 and (g) molds and yeasts - Littman oxgall agar plus 30 micrograms per milli-

liter of streptomycin and 20 units per milliliter of penicillin. All of the media were sterilized by autoclaving for 15 minutes at 121°C. The antibiotics and potassium tellurite were added aseptically after sterilization. The exact composition of the selective media used, along with the methods of counting, has been published by Difco Laboratories, Inc. (1953).

The fluid thioglycollate plates were placed in vacuum dessicators. The air within the dessicators was removed with a water aspirator type of vacuum pump and replaced with natural gas. This was carried out three successive times. The Littman oxgall plates were incubated at 30°C. for three to five days (depending on the rate of mycelial growth), the violet red bile plates at 37°C. for 18 to 24 hours and the rest at 37°C. for 48 hours.

Preparation of microbiological samples

Birds to be used for intestinal samples were sacrificed by dislocating the neck. Individual samples from each of 2 birds from 2 pens on each treatment were used for the microbiological samples. Immediately following the death of the bird, the abdominal cavity was opened and the intestine tied off at the junction of the gizzard and duodenum and at the yolk-sac diverticulum. On the experiment conducted for only

2 weeks, it was necessary to include the tract to 10 or 12 centimeters posterior to the yolk-sac diverticulum to insure a 1 gram sample. This section was then removed and the contents extruded into sterile containers. The entire process took no more than 10 minutes per chick and the samples were immediately taken to the bacteriological laboratory for analysis. All samples were taken at the same time of day (7-9 a.m.) to prevent any possible diurnal variation.

Chemical procedure

The free amino acids of the plasma were determined by column chromotography using the improved chromatographic method of Moore et al. (1958). The acidic and neutral amino acids were determined on a 150 centimeter column maintained at 50°C. by a water jacket with circulating water. The amino acids were eluted from the column with 0.2N sodium citrate buffers of pH 3.25 and 4.25. The basic amino acids were eluted on a 15 centimeter column, also maintained at 50°C., with a 0.35N sodium citrate buffer, pH 5.28.

Two milliliter fractions were collected on a rotating turn-table. The fractions were developed using the technique of Moore and Stein (1954b). One milliliter of a ninhydrin mixture from a 1 liter bottle, stored under nitrogen and wrapped with black tape, was mixed well with each 2 milliliter fraction. These tubes were then boiled for 15 minutes,

cooled until they could be handled, and then 5 milliliters of a 1:1 mixture of ethyl alcohol and water was added to each tube. The tubes were allowed to cool to room temperature and read on a Beckman spectrophotometer, model DU. The readings were made at a wave length of 570 millimicrons with the exception of proline which was read at 440 millimicrons.

The preparation of a leucine equivalent standard curve and all calculations were carried out as described by Moore and Stein (1948, 1954a).

Preparation of blood samples

Blood samples were obtained by heart probe from 4-week-old chicks. Ten milliliters of blood was placed in heparinized tubes. The 10 milliliters of blood was obtained from each of 4 birds from duplicate pens. It was necessary to pool the plasma from 2 birds in the same pen to obtain 10 milliliters of plasma.

The blood proteins were precipitated using 50 milliliters of a 1 percent solution of picric acid per 10 milliliters of plasma as described by Stein and Moore (1954). The resulting precipitate was removed by centrifuging.

Glass tubes containing a 5 to 6 centimeter bed of Dowex 2X-8 resin were used to remove the picric acid. The addition

of 0.02N HCl eluted all the amino acids from the column with the exception of tryptophan, which was retained due to an affinity of the resin for aromatic groups.

The resulting clear solution from the Dowex 2X-8 column was then concentrated under vacuum on a rotary evaporator to 5 milliliters.

The 5 milliliter sample was then brought to a constant volumn of 10 milliliters with 0.02N HCl. This 10 milliliter sample was divided into two 4 milliliter samples and one 2 milliliter sample for analysis. Prior to freezing the samples, the 4 milliliter sample for the 150 centimeter column was adjusted to pH 7-8 by dropwise addition of N NaOH and allowed to stand 4 hours to convert cysteine to cystine. Cysteine is not detected on the column and appropriate calculations were made for its conversion to cystine. After 4 hours, the sample was lowered to pH 2 by dropwise addition of N HCl and all samples were frozen and stored until time for analysis.

EXPERIMENTAL RESULTS

Experiment I

Objective

Arginine was added to casein, zein and casein-zein diets to achieve two levels, 1.2 and 1.8 percent of total dietary arginine, to determine whether growth and feed efficiency was improved and to determine the effect on microorganisms of the intestine.

Method

Day-old crossbred male chicks (Vantress x Arbor Acre) were randomly assigned to triplicate pens of each dietary treatment. The basal diets in Table 1 were used. Feeding and management, and preparation and analysis of microflora samples were as described in Experimental Procedure.

Results

In comparing the performance of chicks, it can be seen in Table 2 that casein with low arginine, and zein with both levels of arginine, produced very poor growth and resulted in poor feed efficiency. The low-arginine casein diet resulted in a weight gain of 20 percent and a feed efficiency gain of about 18 percent over that achieved on the zein diets. However, both diets produced suboptimal weight gains and feed

efficiency. This finding was to be expected, since zein has been reported as being an unbalanced protein which does not support good growth of other species, as well as of chickens. Casein, as discussed earlier, is not a balanced protein for chick growth without the addition of high levels of arginine. Birds on the low-arginine casein diet showed characteristic arginine deficiency symptoms. Combining casein and zein in a proportion to provide arginine at 1.2 percent with protein equal to that in the casein or zein diet produced improved growth and feed efficiency. At a comparable arginine level, 1.2 percent, the diet based on a combination of casein and zein produced weights at 4 weeks of age 31 percent over that of the casein diet and more than 57 percent greater than that achieved on the zein diet. Feed efficiency, while not increased as much as was growth by the combination of proteins, still was improved by some 6 percent over that on a casein diet and 25 percent over that obtained with chicks fed a diet based on zein protein alone. The arginine deficiency symptoms noted in birds on the low-arginine casein diet were not present in the birds on the low-arginine casein-zein diet.

The improvements obtained on the combined protein source were completely overshadowed by the improved growth obtained by increasing the arginine level on the casein diet to 1.8 percent. Not only was the 4-week weight improved nearly 70

percent, but feed efficiency was improved by nearly 30 percent. No birds on the high casein diet showed arginine deficiency symptoms.

The additions of arginine to achieve the 1.8 percent level in the zein and casein-zein diets did not improve growth, but an improvement in feed efficiency was noted in both cases.

Analysis of the intestinal contents of chicks fed diets based on casein, zein and a casein-zein combination showed that coliform and streptococci counts were lowest in the chicks fed casein diets. These counts were considerably higher in chicks on zein diets and still somewhat higher for chicks fed the combination protein source of casein and zein (Figure 1). Chicks fed an unsupplemented zein diet had a lower count in comparison to casein and casein-zein diets in staphylococci, lactobacilli, total aerobes and total anaerobes, but in general, no clearcut effect of protein sources was shown for these microorganisms.

The addition of arginine to all diets increased total counts in all cases except total aerobes, total anaerobes and streptococci on the casein-zein diet. In general, the supplemental arginine increased the microflora population of the intestine of the chick regardless of whether chick growth was increased.

No mold or yeast growth was obtained on the Littman

Table 1. Basal diets for Experiment I

Constituent	Diet (%)		
	Casein	Zein	Casein-zein
Dextrose*	58.7	58.0	59.5
Soybean oil	2.0	2.0	2.0
Cellulose (alphacel)	3.0	3.0	3.0
Vitamin C-19a**	1.0	1.0	1.0
Mineral C-19**	5.3	5.3	5.3
Choline chloride (25%)	1.0	1.0	1.0
Casein	28.0	-	8.5
Zein	-	27.0	18.0
Lysine-HCl (95%)	-	1.3	0.5
DL-trytophan	-	0.4	0.2
Glycine	1.0	1.0	1.0

*The added arginine was compensated for by decreasing the dextrose.

**Vitamin mix C-19a and Mineral mix C-19 are shown in Appendix, Tables 12 and 13.

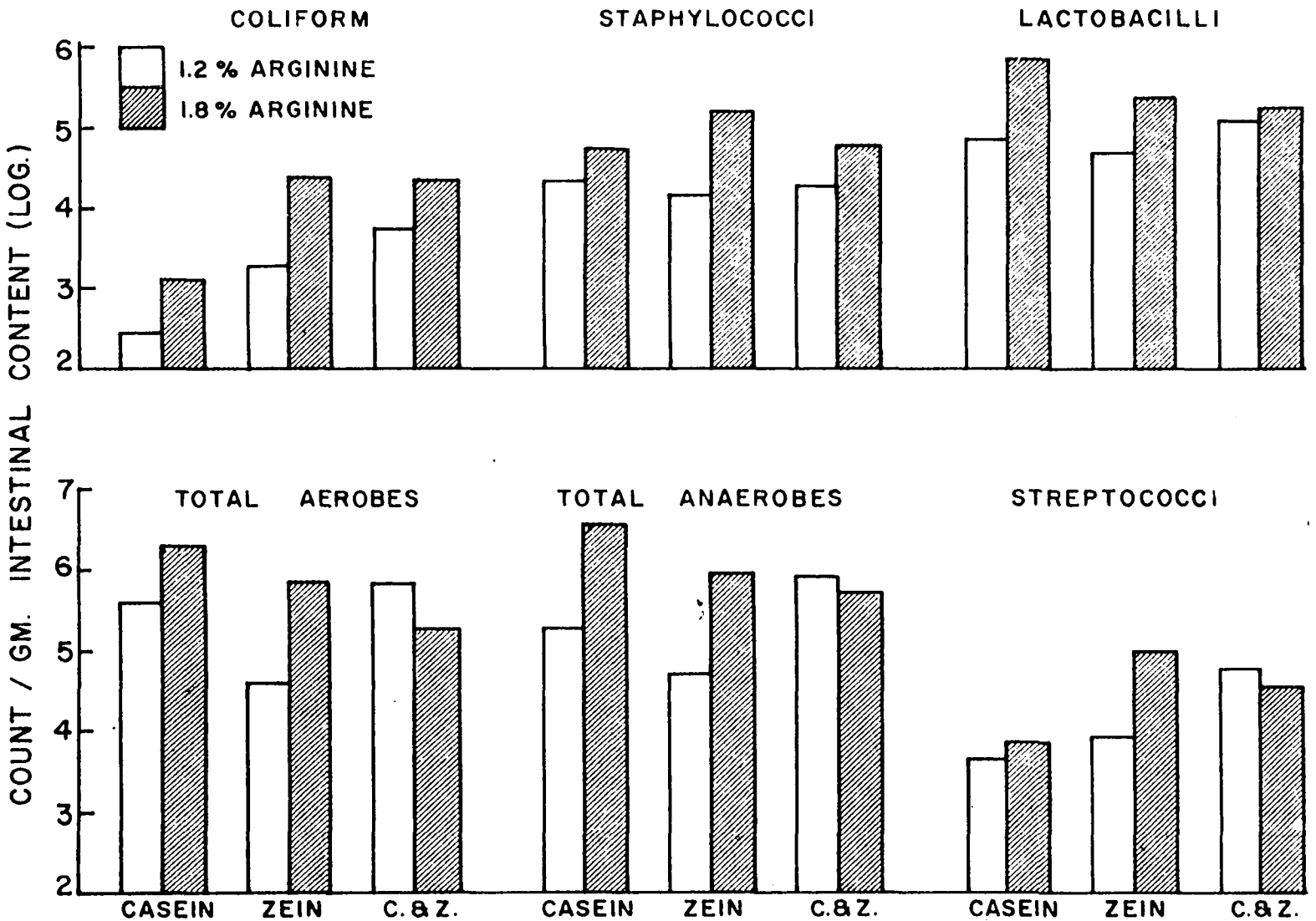
Table 2. Influence of different proteins and an increased level of arginine on chick weight and feed efficiency

Protein source and arginine level in diets (%)	4-week weight[a] (grams)	Feed efficiency[b]
Casein 28.0		
Arginine 1.2	239.9	2.30
" 1.8	404.9	1.72
Zein 27.0		
Arginine 1.2	200.6	2.72
" 1.8	205.7	2.37
Casein 8.5 and Zein 18.0		
Arginine 1.2	315.2	2.16
" 1.8	316.9	2.05

[a]Average per chick of triplicated experimental lots; 5 chicks per lot.

[b]Grams of feed per gram of gain.

Figure 1. Influence of protein source (casein, zein and a combination of casein and zein) plus added arginine on intestinal microflora of the chick.


COLIFORM
STAPHYLOCOCCI
LACTOBACILLI
1.2 % ARGININE
1.8 % ARGININE
COUNT / GM. INTESTINAL CONTENT (LOG.)
6
5
4
3
2
7
TOTAL AEROBES
TOTAL ANAEROBES
STREPTOCOCCI
CASEIN
ZEIN
C. & Z.
CASEIN
ZEIN
C. & Z.
CASEIN
ZEIN
C. & Z.

oxgall agar plates and the coliform counts were lower than the counts of other organisms measured. All organisms measured in this experiment and subsequent experiments were from the anterior portion of the intestinal tract, which may account for the low or absent coliform and mold and yeast counts. Both Johansson et al. (1948) and Lev and Briggs (1956b) reported that the counts in the anterior portion of the intestinal tract of the fowl were very low and quite variable for both coliform and molds and yeast.

Experiment II

Objectives

Three different purified protein sources, which were known to give different growth responses in chicks, were used to note growth differences and also to observe differences in intestinal microflora counts due to protein source and added arginine.

Method

Crossbred male chicks (Vantress x Arbor Acre) were allotted at random to triplicated pens, 5 birds per pen. The basal diets used are described in Table 3. The feeding and management was as described under Experimental Procedure. Intestinal contents were obtained and analyzed as described in Experimental Procedure.

Results

Isolated soybean protein (Drackett Assay Protein, C-1) produced the best weight at 4 weeks with or without added arginine (Table 4). Added arginine did not improve weight of chicks fed this soy protein diet. Casein produced similar weights when the level of arginine was increased to 2.4 percent of the diet as did the soy protein diet. The increased arginine improved the 4-week weights by over 13 percent and improved feed efficiency by slightly over 6 percent when compared to weights and feed efficiency figures obtained on the 1.8 percent arginine, casein diet.

The poorest growth was obtained on the gelatin-casein diet, as expected. The addition of arginine did not improve chick growth on this diet.

Both diets should have contained more than adequate amounts of arginine, based on calculated values, but Drackett protein had a much better balance of amino acids than did gelatin-casein and this was shown by the much superior chick growth.

The only improvement in feed efficiency was, as noted earlier, on the increased arginine level of casein. There was relatively no change in feed efficiency on the Drackett protein diet due to arginine supplementation and the feed efficiency was actually reduced on the gelatin-casein diets

by arginine supplementation.

As was noted in Experiment I, the chick growth did not seem to alter the different microflora counts of the anterior portion of the intestine. There was a difference of 281 grams in the average chick weights at 4 weeks between Drackett protein birds and birds on the gelatin-casein diet, yet there was very little difference in their microflora counts. Comparing the three protein sources, the casein diet appeared to result in slightly lower counts in all organisms than did Drackett protein and gelatin-casein. This was particularly true with both levels of arginine on the counts of lactobacilli, streptococci, total anaerobes and to a certain extent total aerobes.

The arginine level seemed to exert the most influence on the microflora counts of the chick. In comparing the arginine levels of the diet which were formulated to be somewhat similar (2.4 percent on casein, 2.3 percent on Drackett protein, and 2.2 percent on gelatin-casein) it can be seen that with the exception of the coliforms and staphylococci counts, they were all quite similar. The addition of arginine to all diets increased the microflora counts of all organisms measured with the exceptions of coliforms on the Drackett protein diet. The increase noted with increased arginine on the casein diet is of special interest because it occurs even

though a liberal amount of added arginine was necessary to obtain the level of 1.8 percent.

As noted in Experiment I, the coliform counts were low and variable with some plates blank. There were sparse mold and yeast colonies found, but they were very few and variable and are not reported.

Table 3. Basal diets for Experiment II

Constituent	Diet (%)		
	Casein	Drackett protein	Gelatin-casein
Dextrose[a]	58.7	59.5	58.9
Soybean oil	2.0	2.0	2.0
Cellulose (Alphacel)	3.0	3.0	3.0
Vitamin mix C-19a[b]	1.0	1.0	1.0
Mineral mix C-19[b]	5.3	5.3	5.3
Choline chloride (25%)	1.0	1.0	1.0
Casein	28.0	-	5.0
Drackett protein[c]	-	28.0	-
Gelatin	-	-	23.5
DL-methionine	-	0.2	0.1
DL-tryptophan	-	-	0.2
Glycine	1.0	-	-

[a]The added arginine was compensated for by decreasing the dextrose.

[b]Vitamin mix C-19a and Mineral mix C-19 are shown in Appendix, Tables 12 and 13.

[c]Isolated soybean protein, C-1 Assay, The Drackett Co.

Table 4. Influence of three different purified protein sources plus an increased level of arginine on chick growth and feed efficiency

Protein source and arginine level (%) in the diet	4-week weight[a] (grams)	Feed efficiency[b]
Casein		
Arginine 1.8	372.5	1.71
" 2.4	412.2	1.60
Drackett protein		
Arginine 2.3	423.3	1.72
" 2.9	428.4	1.73
Gelatin-casein		
Arginine 2.2	146.8	2.40
" 2.8	142.2	2.58

[a]Average per chick of triplicated experimental lots; 5 chicks per lot.

[b]Grams of feed per gram of gain.

Experiment III

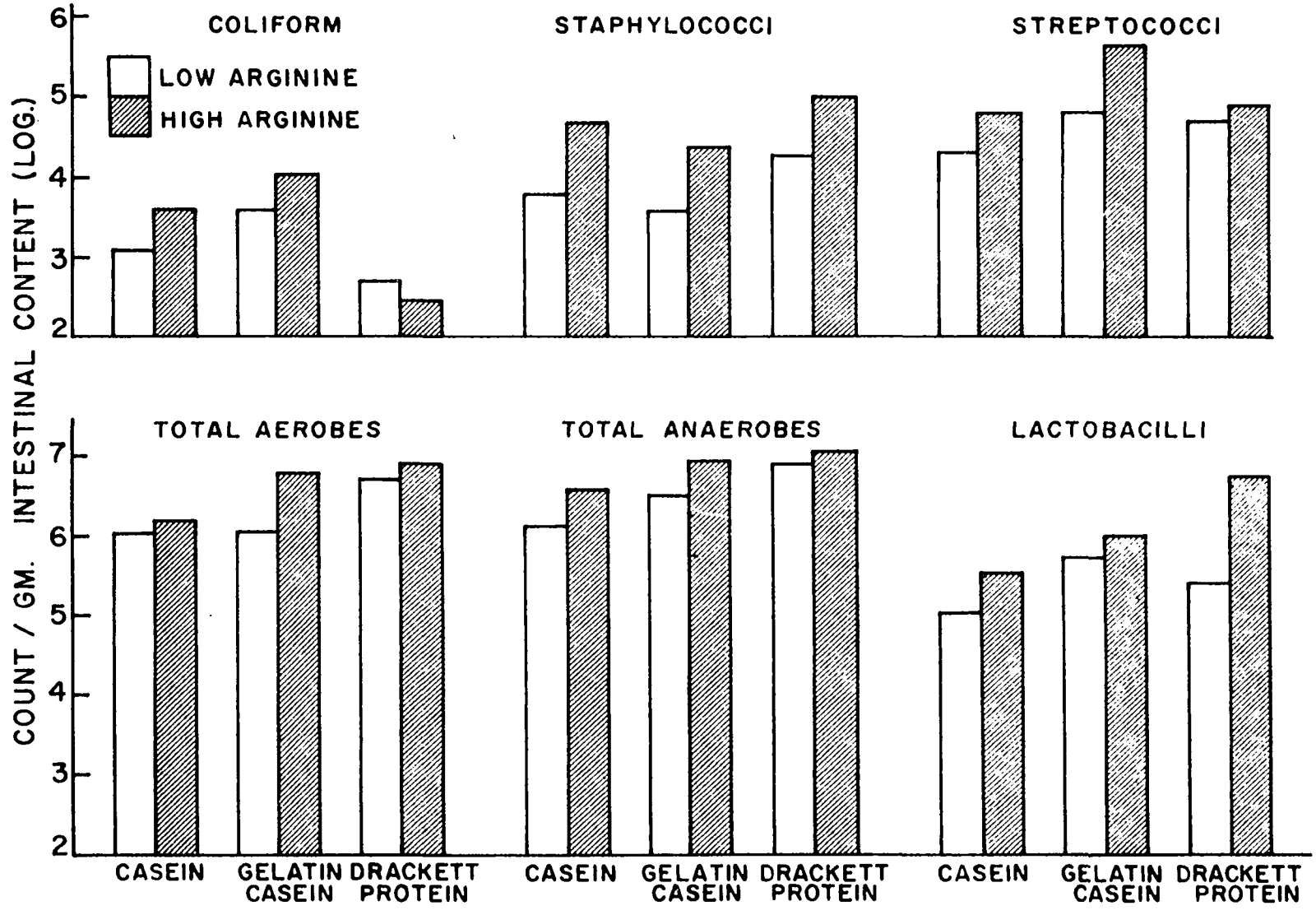
Objective

Experiment III was designed to make further observations on the effect of casein and added arginine on chick growth, intestinal microflora counts and to determine the effects of added arginine on some free amino acids of the blood.

Method

Five male crossbred chicks (Vantress x Arbor Acre) were randomly placed in duplicated pens. The diets of Experiment

Figure 2. Influence of protein source and added arginine on the intestinal microflora.


COLIFORM
STAPHYLOCOCCI
STREPTOCOCCI
LOW ARGININE
HIGH ARGININE
COUNT / GM. INTESTINAL CONTENT (LOG.)
6
5
4
3
2
TOTAL AEROBES
TOTAL ANAEROBES
LACTOBACILLI
7
6
5
4
3
2
CASEIN
GELATIN CASEIN
DRACKETT PROTEIN
CASEIN
GELATIN CASEIN
DRACKETT PROTEIN
CASEIN
GELATIN CASEIN
DRACKETT PROTEIN

I were used as was the feeding and management procedure. Preparation of intestinal contents, blood samples and the laboratory analysis of both were carried out as described in Experimental Procedure.

Results

As can be seen in Table 5, the growth and feed efficiency of chicks fed the casein diet was greatly improved by the addition of arginine to produce a concentration of 1.8 percent of the diet. This was true at both 2 weeks (23 percent) and more so at 4 weeks of age (44 percent). As early as 10 days of age, the chicks on the low-arginine casein diet displayed the typical arginine deficiency symptoms of small spoon-shaped feathers and also broken feathers. A staggering, unsteady gait was also noted somewhat later in the low-arginine chicks and was quite prevalent after 3 weeks of age. The birds receiving high-arginine casein diets exhibited excellent feathering and normal locomotion.

The birds receiving the casein-zein diets all exhibited normal, smooth feathers on both the high- and low-arginine diets. There appeared, however, to be some growth depression on the high-arginine casein-zein diet, not noted in previous experiments. The feed efficiency was also less on this diet, but other than small size, the birds showed no symptoms of

amino acid imbalance.

The birds receiving low arginine on the zein diet made extremely small weight gains and exhibited very poor feathering, not noted in Experiment I. The addition of higher arginine improved the feathering and improved the growth by over 80 percent. However, even with arginine supplementation, the zein diet was ineffective in producing normal growth.

Feed efficiency was improved at both 2 and 4 weeks of age by the addition of arginine on both casein and zein proteins. The poor weight obtained on the high-arginine casein-zein diet was also reflected in the feed efficiency which was less at both 2 and 4 weeks of age.

Microbial counts were made on birds from the casein diets only and they were quite similar to the results found on casein diets in Experiment I (Figure 3). Increasing the dietary arginine content increased all the counts except coliform, which were decreased, and total anaerobes, which were not affected by added arginine. Mold and yeast counts were obtained in this experiment and were also increased by high levels of arginine.

Some very interesting results were revealed in the analysis of some of the free amino acids of chick blood plasma (Table 6). The addition of arginine to the casein diet in-

creased the free arginine blood plasma level as expected; however, this increase was of a very small magnitude. The blood concentrations of free serine, proline and lysine were also increased upon the addition of dietary arginine.

The measurements for lysine in this experiment and subsequent experiments are not corrected for ornithine, which was eluted from the column at the same time as lysine. Cystine is not included in the list of amino acids because it was not detected on the column. The cystine content of casein is very low and is also more difficult to obtain in the sample as described in Experimental Procedure.

The most striking observation in the blood analyses was that the free arginine level of the blood increased very little in comparison to the large growth increase observed in the chicks. A majority of the free amino acids measured in the plasma decreased when arginine was increased in the diet. This would suggest that the growth response obtained with increased additions of arginine to a casein diet is not due necessarily to arginine <u>per se</u>, but may be in part due to an increased utilization of other amino acids also. The absorption into the blood stream of the essential and non-essential amino acids, which are present in excessive amounts in the casein diet for maximum growth of the chick, may be influenced by the addition of arginine.

Table 5. Influence of protein source and an increased level of arginine on chick weight and feed efficiency

Diets	Chick weight[a]		Feed efficiency[b]	
	2-week	4-week	2-week	4-week
Casein				
Arginine 1.2	148.6	315.0	1.79	2.10
" 1.8	184.1	455.0	1.48	1.77
Casein-zein				
Arginine 1.2	151.0	351.0	1.63	2.18
" 1.8	144.9	315.6	1.91	2.40
Zein				
Arginine 1.2	68.0	107.1	4.88	4.14
" 1.8	108.1	194.0	2.08	2.69

[a]Average per chick in grams; two groups of 5 chicks each per experimental treatment.

[b]Grams of feed per gram of gain.

Table 6. The influence of arginine on some free amino acids and ammonia in the blood plasma of young chicks

Substance measured	1.2 percent arginine in diet	1.8 percent arginine in diet	Difference due to added arginine
Aspartic acid	6.43[a]	6.15	- 0.28
Threonine	158.30	120.77	-37.53
Serine	130.02	135.43	+ 5.41
Glutamic acid	17.25	22.16	- 4.91
Proline	159.18	175.78	+16.20
Glycine	55.38	55.75	+ 0.37
Alanine	56.35	43.26	-13.09

[a]Micrograms per milliliter of blood plasma.

Table 6. (Continued)

Substance measured	1.2 percent arginine in diet	1.8 percent arginine in diet	Difference due to added arginine
Valine	69.63	51.68	-17.95
Methionine	20.47	14.38	- 6.09
Isoleucine	29.25	20.21	- 9.04
Leucine	44.15	36.84	- 7.31
Tyrosine	57.57	37.66	-19.91
Phenylalanine	25.18	20.58	- 4.60
Lysine	115.50	121.00	+ 5.50
Histidine	60.90	56.20	- 4.70
Ammonia	21.00	13.60	- 7.40
Arginine	22.30	26.90	+ 4.60

Experiment IV

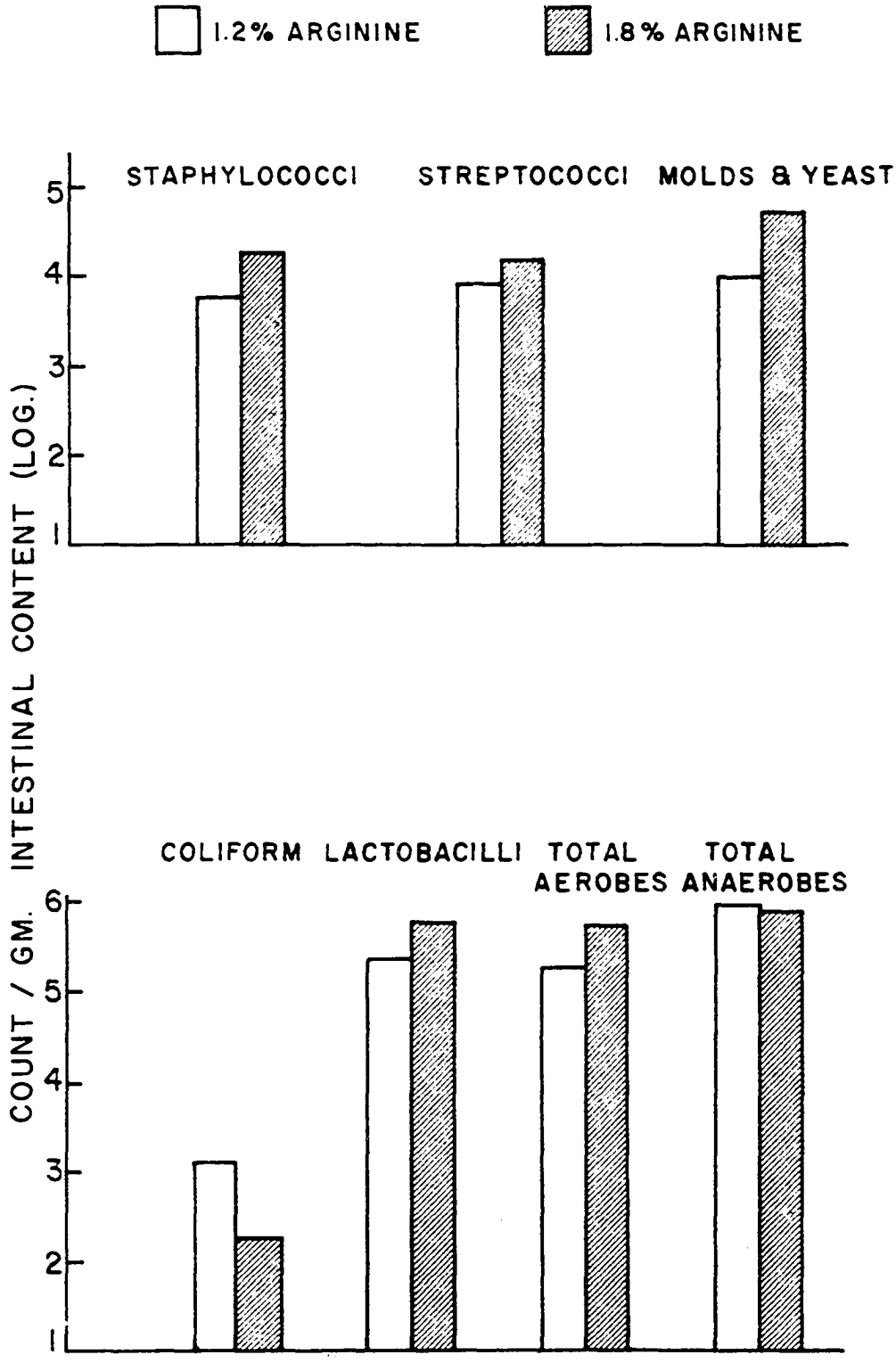
Objective

Earlier experiments at this laboratory indicated a relationship between arginine and lysine in the nutrition of the chick. This experiment was designed to observe the effects of the addition of arginine and lysine to casein and casein-zein diets on chick growth, feed efficiency, external appearance and free arginine and lysine in the blood.

Method

Duplicate pens of 6 day-old male crossbred chicks (Vantress x Arbor Acre) were assigned at random to each

Figure 3. The influence of added arginine on intestinal microflora when added to a casein diet.


1.2% ARGININE
1.8% ARGININE
STAPHYLOCOCCI
STREPTOCOCCI
MOLDS & YEAST
COUNT / GM. INTESTINAL CONTENT (LOG.)
1
2
3
4
5
COLIFORM
LACTOBACILLI
TOTAL AEROBES
TOTAL ANAEROBES
1
2
3
4
5
6

dietary treatment. The same basal rations were used as in Experiment I (Table 1). The diets were calculated to contain 1.2 percent arginine or supplemented if necessary to obtain this level. Arginine at a level of 0.6 percent was then added to obtain the level of 1.8 percent arginine. The lysine levels were calculated to be 2.4 percent on the casein diet and 0.9 percent on the casein-zein diet with supplemental lysine. Lysine was then added to both diets at a level of 0.5 percent lysine to obtain the high level of lysine of 1.4 and 2.9 percent on the casein-zein and casein diets, respectively. Feeding and management was the same as described earlier. Blood samples were obtained and analyzed as described in Experimental Procedure.

Results

The birds on the casein diet containing 1.2 percent arginine began showing typical arginine deficiency symptoms at 8 to 10 days of age. At 10 days, one bird walked with an unsteady gait. Increasing the arginine level to 1.8 percent eliminated the deficiency symptoms and significantly increased (Table 14, Appendix) the weight of the birds (Table 7) at both 2 and 4 weeks of age. ($P = .05$ or less at 2 weeks and $P = .01$ or less at 4 weeks). The feed required per gram of gain was also significantly decreased by arginine additions at 2 weeks ($P = .05$ or less) but no difference was observed

at 4 weeks of age as a result of added arginine.

Additions of lysine to the low-arginine casein diet (1.2 percent arginine) significantly decreased chick weights at 2 weeks ($P = .05$ or less) and also at 4 weeks of age ($P = .01$ or less). This decrease in weight was accompanied by gross symptoms unlike those of the arginine deficiency. Although rough, spoon-shaped feathers from arginine deficiency were evident, the most prominent symptom of the lysine toxicity was the inability of the birds to stand or to walk normally. The symptoms began to appear at 10 days of age with one or two birds having tremors of the legs and the desire to sit more frequently. The chicks were bright appearing and would walk and run when forced. The symptoms became progressively worse and more birds were affected. Their legs trembled severely, they staggered and weaved when walking and by 4 weeks of age all birds in one pen were prostrated. In the other pen fed the high-lysine, low-arginine diet, birds staggered when forced to move. They looked very rough and emaciated at this time because of their inability to get to the feeders to eat.

There was a statistically significant increase in feed required per gram of gain ($P = .05$ or less) at 2 weeks but this could not be shown at 4 weeks due to a high variation be-

tween duplicate pens. This variation was accounted for by the fact that some birds could get to feed and others could not.

Supplementing the diet with both arginine and lysine at the same levels used alone seemed to counterbalance the effects of each used alone. Whereas at both 2 and 4 weeks, arginine supplementation significantly increased weight gains and prevented arginine deficiency symptoms, and extra lysine added to the already high amount contained in casein produced definite toxicity symptoms; when both arginine lysine were added, neither of these phenomena were observed. The arginine apparently was not effective in improving weight gains in the presence of the excess lysine and the lysine toxicity was apparently offset by the extra arginine added. At 2 weeks, birds on the diets supplemented with both arginine and lysine appeared small and rough, but they did not exhibit arginine deficiency symptoms or lysine toxicity symptoms. Their legs appeared sound and no quivering, staggered gait was noted at this time.

At 21 to 23 days of age, 2 or 3 birds on the high-arginine, high-lysine diets began to show signs of leg weakness as noted earlier in birds fed the diets high in lysine alone. By the end of the experiment (28 days), 4 birds out of the 2 pens showed definite symptoms.

There was no particular trend noted in feed efficiency as a result of feeding the high-arginine, high-lysine diets, feed efficiency for these lots being intermediate between that of lots fed the arginine-supplemented and the lysine-supplemented diets and not being significantly different from birds fed the unsupplemented basal diets.

Table 7. Chick growth and feed efficiency as affected by additions of arginine and lysine to a casein diet

Modification of basal diet	Av. chick weight[a] (grams)	Feed efficiency[b] (grams)
	2-week	2-week
None	149.4	1.58
Arginine	178.8	1.50
Lysine	109.7	2.00
Arginine x lysine	152.6	1.58
	4-week	4-week
None	290.1	1.72
Arginine	391.1	1.74
Lysine	134.6	3.65
Arginine x lysine	319.8	1.88

[a]Average per chick of duplicate experimental lots; 6 chicks per lot.

[b]Grams of feed per gram of gain.

Supplementation of the casein-zein diets with arginine and lysine, in contrast to supplementation of casein diets, had a markedly different effect (Table 8). The zein, being deficient in lysine, reduced the calculated lysine content

of the diet from 2.4 percent to 0.9 percent, while the arginine content of the diet was maintained at 1.2 percent.

The birds on the casein-zein basal did not show any arginine deficiency symptoms. The addition of arginine did not improve the chick weights at either 2 or 4 weeks of age. However, arginine significantly increased the feed required per unit gain at 2 weeks ($P = .05$ or less) and 4 weeks ($P = .01$ or less) and did depress growth slightly in comparison to the birds on unsupplemented diets.

The addition of lysine (0.5 percent) to this diet did not evoke any toxicity symptoms. Lysine supplementation improved the weight of the birds significantly at 2 weeks of age ($P = .05$ or less) but the increase noted at 4 weeks of age was not statistically significant (Table 14, Appendix). Feed efficiency was significantly improved at 2 weeks of age ($P = .05$ or less).

Addition of both arginine and lysine did not alter the chick weights or feed efficiency to any significant extent over birds fed unsupplemented diets. The combination of casein and zein apparently altered the amino acid composition of the diet and influenced the response of the chicks to the supplementation of arginine and lysine.

In general, performance of chicks achieved on the casein-

zein diet (Table 8) did not equal that achieved on the casein diet (Table 7) when the casein diet was supplemented with arginine. However, it can be noted in Table 8 that the average 4-week weights of the birds receiving both arginine and lysine are depressed slightly in comparison to the unsupplemented birds and also feed required per unit of gain is increased. This is similar to the growth depression on this diet noted earlier due to arginine addition alone.

Tables 9 and 10 show the effects of added dietary arginine and lysine on the blood concentrations of some free amino acids when the arginine and lysine were added to casein and casein-zein diets, respectively.

The analyses concerning the effects of arginine and lysine supplementation on the free amino acids of the blood are given in Table 15 (Appendix). The addition of arginine to the casein diet did not significantly increase the amount of free arginine in the blood, although the growth of the birds was increased considerably. This substantiates the data of Experiment III in which a small but consistent increase in free arginine in the blood was found in birds on casein. This small increase appears insignificant when compared to the growth it produces on the birds.

There was no significant change in lysine, histidine and ammonia by increased arginine, although on the low-lysine

diet, there was an increase in blood lysine concentration of some magnitude which agrees with the increase in lysine noted in Experiment III.

Additions of lysine to the casein diet significantly increased the lysine content of the blood (P = .05 or less), but did not significantly affect the amounts of arginine, histidine or ammonia. Histidine was increased slightly, but this could partially be explained by the fact that histidine emerges from the column very close to lysine. When lysine was increased, the significantly increased amount present in the blood made the differentiation of histidine difficult and may have in this way biased the amount of histidine measured to some extent.

Table 10 shows the effects of added arginine and lysine to a casein-zein diet. Although growth was not materially affected by additions of arginine, lysine or a combination of the two amino acids, the levels in the blood were affected. Another interesting comparison can be made between casein and casein-zein diets in the amount of free arginine found in the blood. Although added arginine did not improve growth on the casein-zein diet, the arginine content of the blood was more than doubled when dietary arginine was increased from 1.2 to 1.8 percent. This increase in arginine was statistically significant at the .01 level of probability. The level of

24.4 micrograms of free arginine per milliliter of plasma was also almost double the amount found in chicks fed the casein diet.

There was a significant interaction noted with additions of arginine and lysine to the casein-zein diet (P = .01 or less) in relation to free lysine of the blood (Table 10). The interaction appears to arise from the fact that at the lower lysine (0.9 percent) added dietary arginine increased free blood plasma lysine, while at the higher dietary lysine

Table 8. Chick growth and feed efficiency as affected by additions of arginine and lysine to a casein-zein diet

Modification of basal diet	Av. chick weight[a] (grams)	Feed efficiency[b] (grams)
	2-week	2-week
None	136.8	1.57
Arginine	135.5	1.75
Lysine	169.5	1.39
Arginine x lysine	161.5	1.87
	4-week	4-week
None	304.2	1.98
Arginine	287.3	2.15
Lysine	331.1	2.00
Arginine x lysine	292.8	2.29

[a]Average per chick of duplicate experimental lots; 6 chicks per lot.

[b]Grams of feed per gram of gain.

Table 9. Influence of added arginine and lysine to a casein diet on some free amino acids and ammonia in the blood

Lysine in diet (%)	Substance measured	Arginine in diet (%)	
		1.2	1.8
2.4	Lysine	138.4[a]	160.1
	Histidine	69.6	68.2
	Ammonia	19.0	18.0
	Arginine	12.6	14.6
2.9	Lysine	183.4	176.6
	Histidine	75.2	95.5
	Ammonia	15.8	19.0
	Arginine	11.9	17.0

[a]Micrograms per milliliter of blood plasma.

Table 10. Influence of added arginine and lysine to a casein-zein diet on some free amino acids and ammonia in the blood

Lysine in diet (%)	Substance measures	Arginine in diet (%)	
		1.2	1.8
0.9	Lysine	45.8[a]	67.9
	Histidine	28.8	28.6
	Ammonia	14.4	13.2
	Arginine	24.4	58.1
1.4	Lysine	108.0	75.6
	Histidine	39.5	30.7
	Ammonia	18.9	14.8
	Arginine	20.0	40.9

[a]Micrograms per milliliter of blood plasma.

(1.4 percent) added arginine resulted in decreased free blood plasma lysine, although the amount is still higher than that found in chicks fed the unsupplemented diet. This significant interaction was not observed on the casein diet, although Table 9 shows that a similar but less significant pattern occurred. At the lower lysine level, added arginine increased blood lysine by 15 percent while at the higher lysine level, added arginine slightly decreased blood lysine.

The addition of lysine itself to the casein-zein diet did significantly increase the lysine level of the blood ($P = .01$ or less), the increase being over 100 percent on the low-arginine diets. It also significantly decreased the amount of free arginine in the blood ($P = .01$ or less) on both low and high dietary arginine levels.

The blood concentrations of histidine and ammonia were not appreciably altered by additions of arginine, lysine or a combination of the two.

Experiment V

Objective

This experiment was designed to further observe the effects of the diets used in Experiment IV on chick growth, feed efficiency and intestinal microflora.

Method

Six 1-day-old crossbred male chicks (Vantress x Arbor Acre) were randomly assigned to each of duplicate pens. The casein and casein-zein diets in Table 1 were used, and the feeding and management was as described in Experimental Procedure. All microbiological samples were obtained and analyzed according to methods described earlier with the exception that three birds from one pen on each treatment were used for the analyses.

Results

The growth, feed efficiency and appearance of the birds in this experiment were very similar to those in Experiment IV. The addition of arginine to maintain a 1.9 percent dietary level on a casein diet improved the growth and feed efficiency (Table 11 and Table 16, Appendix) and prevented the ragged feathering observed of the low-arginine birds. The addition of lysine to 2.9 percent of the casein diet caused a decrease in growth and poor feed efficiency as was true also in Experiment IV. A stilted gait and inability to maintain balance began to appear in some of the chicks at 10 days of age. At 2 weeks, arginine supplementation improved chick weights ($P = .01$ or less). As in Experiment IV, arginine supplementation of the casein diet receiving supplemental lysine counteracted the effects of excessive lysine.

Arginine supplementation of the casein-zein diet improved weight gains only slightly. This basal contained 1.2 percent arginine.

As in Experiment IV, the addition of lysine on the casein-zein basal to increase the lysine from 0.9 percent to 1.4 percent lysine did slightly increase the average chick weight. The addition of both lysine and arginine produced weight gains about equal to those of chicks fed the basal with lysine supplementation.

Table 11. The influence of protein source and added amino acids on 2-week chick weights and feed efficiency

Protein source and amino acid treatment	Chick weight[a]	Feed efficiency[b]
Casein		
None	120.7	2.13
Arginine	170.3	1.88
Lysine	110.8	2.21
Arginine x lysine	148.3	1.60
Casein-zein		
None	146.5	1.66
Arginine	149.7	1.74
Lysine	156.8	1.77
Arginine x lysine	153.2	1.82

[a]Average per chick mgrams; two groups of 5 chicks each per experimental treatment.

[b]Grams of feed per gram of gain.

Feed efficiency was not affected greatly by amino acid additions. Somewhat poorer efficiency was evident when arginine and lysine were added either alone or in combination.

Figures 4 and 5 show the effect of arginine and lysine additions on the intestinal microflora of the chicks. In comparing the two figures, there was not a great deal of difference between the casein and casein-zein protein sources as to effects on intestinal flora. Staphylococci, streptococci, mold and yeasts and coliform were higher in general on the casein-zein diets than on the casein diet. The other counts were quite similar.

In comparing intestinal microflora count changes due to arginine additions on the two different proteins, it can be seen that counts were generally lower with the arginine supplemented diets. This was not noted in earlier experiments. This was particularly true of lactobocilli and streptococci on diets of both protein sources. This difference may be explained in part by the age of the birds. All previous microflora counts were made on birds 4 weeks of age, while the birds on Experiment V were only 2 weeks old.

With few exceptions, lysine appeared to increase the microflora counts on both diets. Arginine and lysine supplementation, in general, seemed to result in counts equal to or higher than the highest counts obtained by the addition of

Figure 4. The influence of added arginine and lysine to a casein diet on intestinal microflora

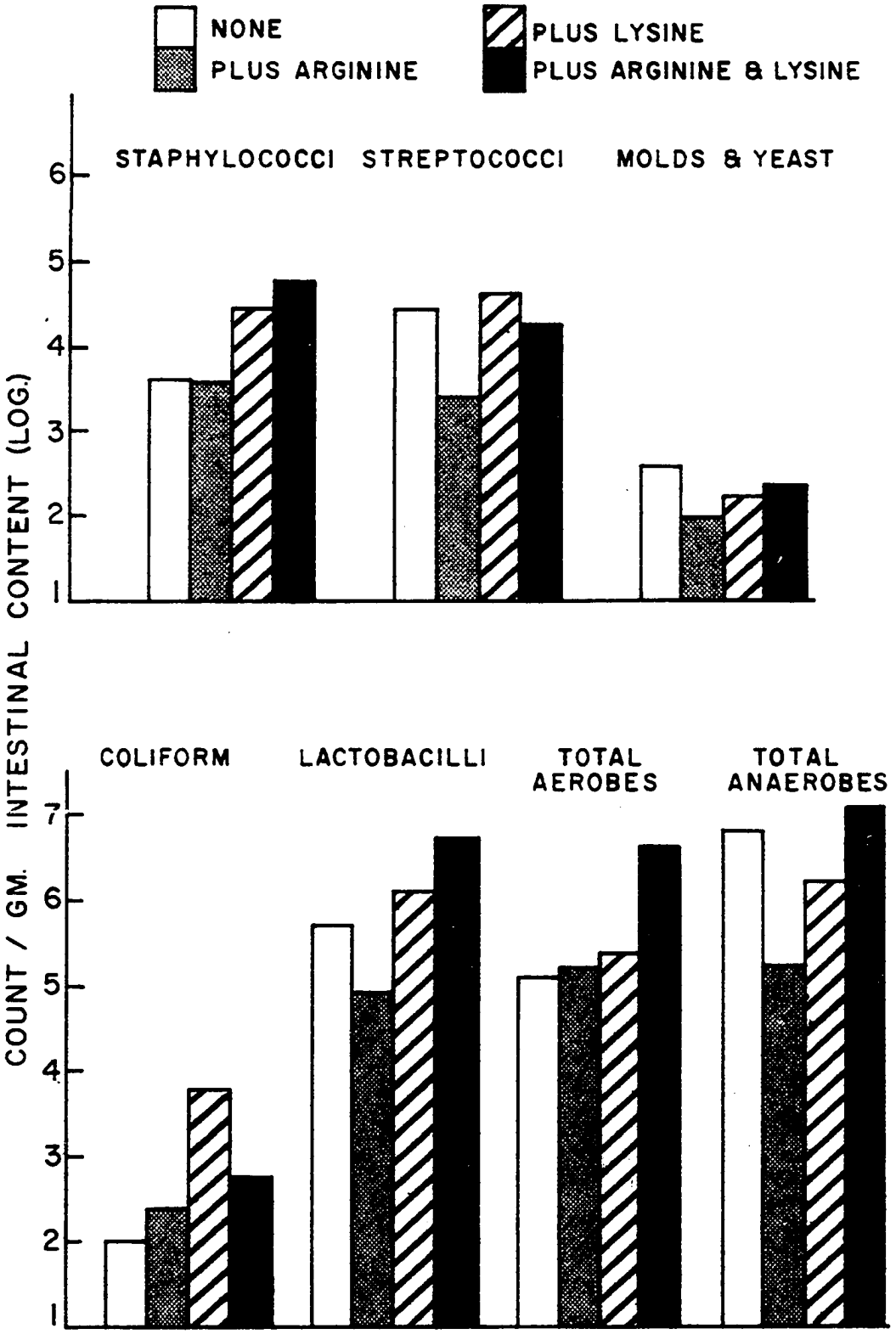
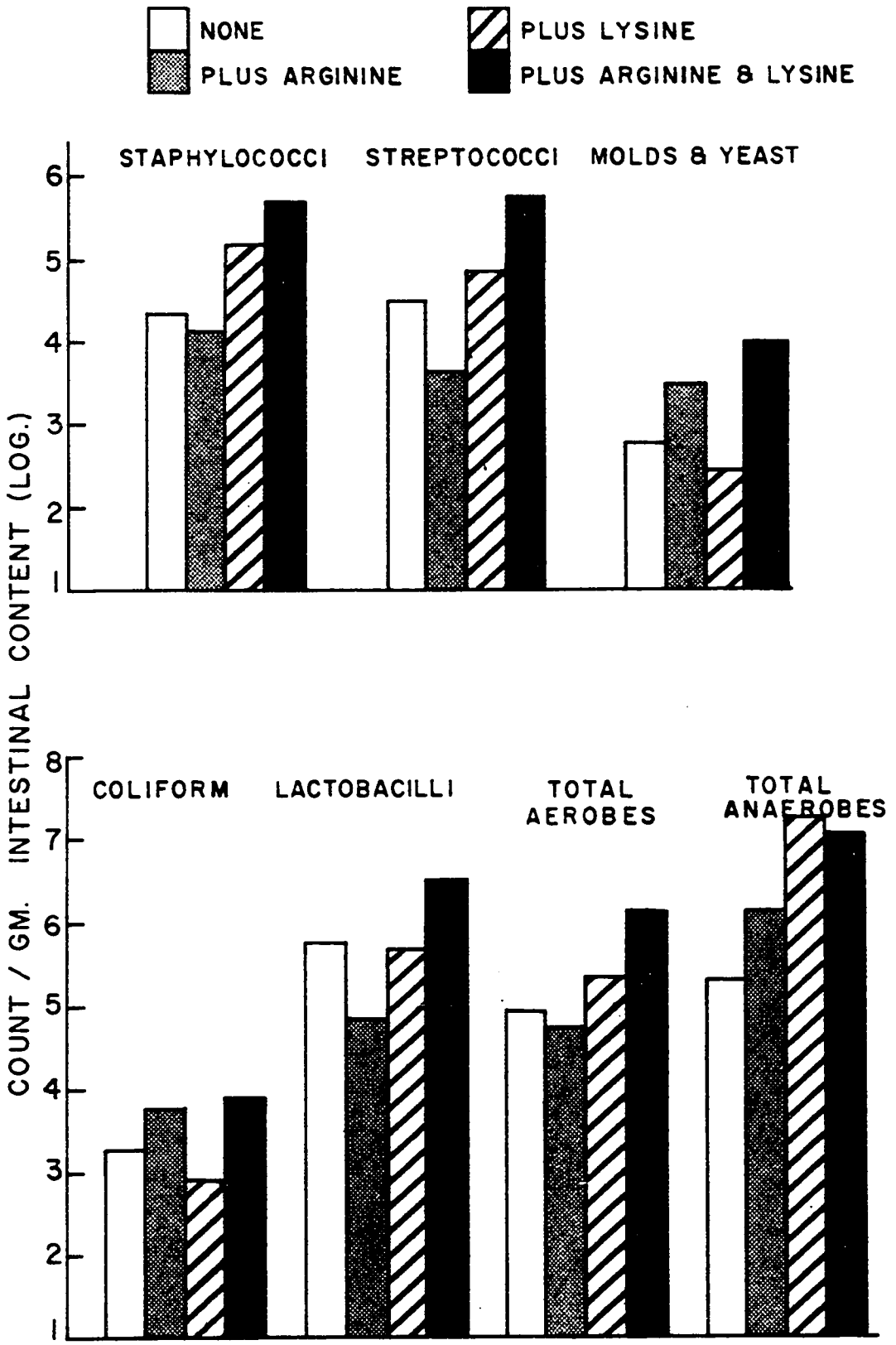

NONE
PLUS ARGININE
PLUS LYSINE
PLUS ARGININE & LYSINE
STAPHYLOCOCCI
STREPTOCOCCI
MOLDS & YEAST
COUNT / GM. INTESTINAL CONTENT (LOG.)
1
2
3
4
5
6
7
COLIFORM
LACTOBACILLI
TOTAL AEROBES
TOTAL ANAEROBES

Figure 5. The influence of added arginine and lysine to a casein-zein diet on intestinal microflora.


NONE
PLUS ARGININE
PLUS LYSINE
PLUS ARGININE & LYSINE
STAPHYLOCOCCI
STREPTOCOCCI
MOLDS & YEAST
COUNT / GM. INTESTINAL CONTENT (LOG.)
1
2
3
4
5
6
COLIFORM
LACTOBACILLI
TOTAL AEROBES
TOTAL ANAEROBES
1
2
3
4
5
6
7
8

either amino acid alone. This was true of all counts made except of streptococci and coliforms on the casein diet.

DISCUSSION

Arginine and Lysine Supplementation

Over a period of the last 5 to 8 years, amino acid supplementations of purified and also practical diets have received a great deal of interest. This is particularly true in the case of arginine, which has been shown to be required by chicks on purified diets in higher amounts than on practical rations, and lysine, which has been shown to be involved with arginine in the chick's requirement for each.

Many possible explanations of the high-arginine requirement of chicks on purified diets have been advanced. Increased muscle creatine synthesis has been given as a possible reason for the high-arginine requirement of birds on semi-purified diets (Griminger et al. 1955) and also the presence of creatine in natural feed ingredients has been postulated to exert a sparing action on arginine in practical rations (Snyder et al. 1956). Unidentified factors in corn which enhance the utilization of arginine on practical diets has also been offered as an explanation (Krautmann et al. 1957), as well as the more rapid absorption of free arginine and its rapid breakdown to urea in birds on casein diets (O'Dell et al. 1958).

The last year or so another aspect has received more attention in the literature; that is, the amino acid compo-

sition of protein itself and amino acid imbalances in the diets of birds and animals. Amino acid composition of casein has been suggested as responsible for the high arginine requirement of chicks on casein diets (Anderson and Dobson 1958). Also, chicks receiving a free amino acid diet formulated to duplicate the amino acid makeup of casein required 2.06 percent arginine for maximum growth in the experiments of Klain et al. (1959). More recently the high lysine level of casein rations was reported as being most responsible for the higher arginine requirements of chicks on this diet (Anderson and Dobson 1959). A relationship has been noted between arginine and lysine, particularly in growth, feather depigmentation and tyrosinase activity of birds on diets containing casein-zein as a source of protein (Owings and Balloun 1959).

Arginine supplementation of casein diets resulted in increased chick weight gains and improved feed efficiency in the presently reported experiments. These findings are in agreement with the many reports noted in the Arginine Requirement section of the Review of Literature.

The addition of arginine to zein and casein-zein diets did not produce any increase in growth. This could be explained on the basis of different amino acid composition of the two protein sources. Some of the essential and non-

essential amino acids in the casein diet are present in excessive amounts for optimum chick growth. This could account for the high requirement of arginine on this diet. The excellent growth obtained on the isolated soybean protein (Drackett Assay Protein, C-1) was not improved by the addition of arginine nor was the poor growth of the birds on the zein and gelatin-casein diets. Zein and gelatin both represent very insoluble proteins, which also contain a very poor amino acid balance in terms of the requirement of the chick. Drackett protein has a more favorable amino acid balance.

Lysine addition to the casein diets caused toxicity symptoms in the chick at levels much lower than previously reported. The calculated level of lysine in this experiment was 2.9 percent, compared to 4.0 percent as was found toxic by Anderson and Combs (1952).

The addition of arginine in combination with lysine tended to counteract the effects of the lysine toxicity on the casein diets for a period of time, but by the end of the third week the birds were beginning to show toxicity symptoms compared to 10 days on a diet with high lysine alone. The amount of lysine in the blood of the 4-week-old chick receiving arginine as well as lysine was only 7 micrograms per milliliter less than those receiving supplemental lysine alone, but this was apparently sufficient to largely elim-

inate the lysine toxicity symptoms.

Table 6 presents a summary of analyses of some of the free amino acids of the blood from chicks fed a casein diet. The addition of arginine to the casein diet to increase its level to 1.8 percent altered the amount of free arginine in the blood by less than 5 micrograms per milliliter. This small increase in free plasma arginine does not seem large enough to account for the tremendous increase in chick growth noted from the increase in dietary arginine (Table 5). However, it can be seen in Table 6 that a majority of the other amino acids are decreased in the blood plasma by the dietary addition of arginine. In the case of threonine, this reduction amounts to over 37 micrograms per milliliter of plasma or a decrease of 23 percent.

The reduction in many amino acids of the blood plasma may in part explain the statements made by Krautmann et al. (1958). These authors felt that the growth response due to the addition of practical ingredients to a casein diet was more vitamin-like than protein or amino acid. It is evident from this experiment that the addition of arginine per se is not the only reason for the improved weight gains of the chick. Added arginine apparently significantly affects the absorption of other amino acids and this may greatly affect the overall nutrition of the chick. The addition of arginine balances

the amino acids absorbed in such a way that the excess of certain amino acids present in casein are not absorbed from the gut and a more balanced amino acid picture is thus present in the blood. This may in part, explain the general increase in microflora population of the gut when arginine is added to the diet. The excess amino acids not absorbed are therefore available in the intestinal tract to the microflora.

The amino acids that compose the protein molecule of casein may be released in the gastrointestinal tract at different rates and to different extents when arginine is added to the diet. However, Denton and Elvehjem (1953) reported that the rate and extent of liberation of amino acids from beef, casein and zein were approximately the same. Arginine was reported by the authors to be liberated very rapidly from all three protein sources. It also is interesting to note that lysine was liberated very slowly from casein in their study.

The in vivo study of Donovan (1955) showed that the availability to the chick of the threonine, lysine, histidine and isoleucine in casein appeared to be less than the availability of leucine, arginine and phenylalanine, in contrast to the in vitro studies with casein by Denton and Elvehjem (1953). Donovan also noted that the free amino acids added to the diet are utilized to a much greater extent than those supplied in protein-bound form, and the addition of 0.38 percent arginine

to a diet in which the protein source was composed of isolated soy protein and casein appeared to decrease slightly the utilization of the other bound amino acids. However, in general, he found that arginine, lysine or methionine added to a chick's diet enhanced the utilization of protein-bound amino acids.

Another factor which could be involved in the differences of the amino acid blood values of the diets used in these studies is their absorption at different rates and to different extents from the intestinal tract. Some *in vitro* work with isolated sections of rat intestine (Pinsky and Geiger 1952) have shown that a competition may exist between amino acids and chemically similar substances for the selective processes of absorption or concentration.

Very little work has been done in measuring the amino acids in the urine of the chick; however, some of the variation noted in the amino acids of the blood plasma may be due to excretion in the urine. O'Dell *et al.* (1960) determined that the major amino acids found in the urine of the growing male chicken, in order of decreasing concentration, were glycine, proline, glutamic acid, hydroxyproline, aspartic acid, lysine, ornithine and arginine.

Still another factor which could be involved is the removal of the amino acids from the blood of the chicken by

various tissues at different rates and to different extents. The alteration of the concentration of various amino acids in the blood by the addition of arginine and lysine could alter the utilization of the amino acids by tissues.

The relation of lysine and arginine noted at this laboratory by Owings and Balloun (1959) can be explained to a certain extent by the free amino acids as measured in the blood in these trials. As the level of arginine was increased in the casein-zein diet, the free arginine of the blood increased from an initially high level in comparison to the free arginine level of birds on the casein diet to an even higher value (Table 10). This increase in arginine of the blood plasma was accompanied by an increase in the concentration of free plasma lysine. The levels of plasma arginine and lysine were also quite high when the diet was supplemented with both arginine and lysine. The increase in free lysine of the blood plasma would account for the significant increase in tyrosinase activity, noted by Owings and Balloun (1959), and also the decrease of depigmentation noted in feathers of birds on low-lysine, high-arginine diets. The addition of dietary arginine would increase the amount of lysine available for pigmentation. The blood plasma arginine levels were not as high on the casein diets as noted on the casein-zein diets, but there was also an increasing plasma lysine concentration when arginine was added to the diet.

The increase of free lysine in chick blood plasma when arginine was added to the casein diet is very hard to explain, particularly since there is an excess of lysine in casein, in terms of the requirement of the chick. However, Donovan (1955) pointed out that lysine was one of the amino acids in casein which appeared to be less available to the chick, which may account for its increase with arginine supplementation.

The addition of lysine to both casein and casein-zein diets decreased the amount of free arginine in the blood. This reduction in plasma arginine by increased dietary lysine was also reported by Dunkelgod *et al.* (1960). They noted that with a uniform rate of gain, as dietary lysine was increased on a 38 percent casein diet, the levels of arginine, aspartic acid, asparginine and glycine in the blood plasma were decreased. At the same time, they noted an increase in free lysine, isoleucine and histidine of the blood plasma. This work was conducted with Broad Breasted Bronze turkeys.

Roth and Allison (1949) reported the addition of 4.8 percent glycine or 4.8 percent glycine plus 1.7 percent L-arginine to a casein diet containing excess methionine counteracted the weight loss and in part the kidney hypertrophy caused by excess methionine.

The antagonistic effect noted in *Neurospora* between

arginine and lysine (Doermann 1944) and the antagonistic action of excess leucine on isoleucine on low-protein diets in the rat (Harper et al. 1955) also point out some very important relationships of amino acids which may be involved in the absorption and utilization of amino acids by the chick.

The interrelationships of amino acids noted in this experiment and in reports of other experiments indicate the importance of these relationships to growth and other criteria of physiologic response.

Fisher et al. (1960) recently advanced a theory in relation to the high arginine requirement of chicks on casein diets based on experiments conducted at their laboratory. They postulated that addition of arginine to a casein diet was a correction of the optimum ratio for essential limiting amino acids to total effective protein (or essential amino acids). Thus, optimum growth would not be obtained until arginine was supplemented, not only to meet the arginine requirement of the chick (1.2 percent arginine) but also to balance the excess of other essential amino acids.

The addition of 0.2 percent lysine to a diet based on peanut meal (Richardson et al. 1953) which was deficient in both lysine and methionine, increased the concentration of blood plasma arginine, lysine and valine but did not change that of methionine. The addition of 0.4 percent dietary

methionine alone increased the concentration of arginine, lysine and valine in the blood plasma of chicks.

Work by Dunkelgod et al. (1960) and results from these experiments are in disagreement with the results of Richardson et al. (1953). Dietary lysine additions in both these experiments and those of Dunkelgod et al. (1960) resulted in decreased free arginine in the blood. However, it should be noted that the peanut meal ration of Richardson et al. (1953) is deficient in lysine which might influence the amino acids found in the blood.

Ammonia was included in the tables of this experiment with the amino acids to emphasize any change in its concentration due to dietary supplementation with arginine and lysine. However, its presence must be qualified by reference to the work of Conway and Cooke (1939) which showed that ammonia cannot be detected in fresh-shed blood. The ammonia in fresh blood appears within the first several minutes due to the deamination of adenine, guanine, guanosine, cytosine, and cytidine.

The addition of arginine to a casein-zein diet also increased the free lysine of blood plasma, which explains the improved pigmentation noted by Owings and Balloun (1959), when arginine was added to a diet deficient in lysine. They

also noted a significant increase in tyrosinase activity with high levels of both arginine and lysine. This can be explained on the basis of the significant interaction of lysine and arginine dietary additions on the free lysine in the blood. The addition of arginine would increase the amount of lysine available for pigmentation. It was also noted on the casein-zein diets that the addition of lysine to the diet significantly decreased the amount of free arginine in the blood.

Alteration in Microbial Population

The additions of the amino acid arginine to diets of varying protein generally increased the microbial counts of the intestine (Figures 1 and 2). The increased microbial counts occurred with diets that varied greatly in their ability to support good chick growth. Diets based on zein and gelatin as protein sources supported very poor chick growth, but various microbial counts of chicks fed these diets were little different from those of chicks fed protein sources which supported very good growth. Thus, the altered microflora cannot be explained on the basis of rapidity of chick growth, as in Experiments I and II, the increase in microbial population was also noted in Experiment III when arginine was added to the basal casein diet.

Very low counts were obtained in coliforms in the anter-

ior portion of the intestine studied. This is in agreement with Johansson et al. (1948) and Lev and Briggs (1956b) whose reports show increasing microbial counts from the duodenum to the large intestine. Their coliform counts were extremely low in the duodenum and upper part of the small intestine which was also true in these experiments.

The mold and yeast counts in these experiments were also variable and sometimes undetectable. This is very likely due also to the intestinal portion examined. Mold and yeast counts, as well as coliform counts, are much higher and less variable from the cecas or large intestine than from the anterior portion of the intestine (Johansson et al. 1948).

The variation in the counts made analyses of these data very difficult. However, the consistency noted in Experiments I, II and III in relation to increased counts due to the addition of arginine is of importance to note. Another point to note is that the increased dietary arginine concentration in the case of casein diets was made in addition to the arginine necessary to bring the level up to the experimental level. In one case this addition was made to bring the arginine level up to 2.4 percent and from 1.8 percent, both levels requiring liberal arginine supplementation.

Figures 4 and 5 show microbial counts from birds on casein and casein-zein diets from Experiment V. They are

from birds that are 2 weeks old, while in the earlier experiments discussed, the birds were 4 weeks of age. To utilize the available laboratory facilities it was necessary to terminate the experiment at this time.

The smaller birds dictated the necessity of taking a larger portion of the intestine as discussed in Experimental Procedure. This may explain the increased counts obtained from coliforms and molds and yeast in contrast to earlier experiments discussed where chicks were 4 weeks of age and only the most anterior portion of the small intestines were sampled for microbial populations.

In viewing Figures 4 and 5, a general trend is evident towards higher microbial counts with the dietary additions of arginine, lysine and a combination of both, with a few exceptions. The effects of arginine addition appeared more variable in this experiment than noted before. This may be due to the age of chicks and sampling technique or to variation alone, but it is reasonable to believe that trends would be more firmly established if chicks had been on experimental diets for a longer period of time.

These data are somewhat in contrast to the findings of Anderson, Cunningham, and Slinger (1952). They reported a failure of increasing protein levels (17 to 26 percent) to

alter intestinal microbial counts. They did note some increase in anaerobes and microaerophiles which generally agrees with the increases noted in total anaerobes in these experiments. They also reported increases in chick growth with increasing protein levels up to 23 percent.

The toxic effects of added lysine and the deficiency effects of low-arginine on the casein diet did not seem to be reflected in the intestinal microbial counts.

Quinn et al. (1953) concluded from their work with young pigs that there is no "normal flora" in the pig. They suggest that the number and type of organisms is influenced by the amount and quality of the ration consumed and that averages mean little except in relation to a specific ration.

This statement would very likely apply also to the chick as supported by work reported by Reyniers et al. (1949) at the LOBUND Laboratory. They have been successful in rearing germ-free White Wyandotte Bantam chicks through an entire life cycle and into a second generation.

Donovan (1955) expressed the opinion that the microorganisms within the small intestine might be influenced by diet to predigest the protein in the diet, thus making it more available to proteolytic enzymes. A somewhat analogous situation is the improvement noted in in vitro cellulose digestion by rumen microorganisms when amino acids were added

to the basal diet (Trenkle, 1958). In the presence of a readily available source of nitrogen (urea) the addition of alanine and proline improved cellulose digestion during the early part of the fermentation (before 20 hours) and methionine improved cellulose digestion for the entire 24 hour digestion period.

The reason for the increased microflora in these experiments, as indicated in the earlier discussions, may not be due necessarily only to the arginine or lysine addition to the diet, per se. It was demonstrated in Experiment III that arginine decreases the amounts of many of the amino acids in the blood plasma. It is possible then that if these amino acids were unabsorbed they would be available to the microflora of the intestine and one or all could be responsible for the increase in the microflora population.

SUMMARY

The addition of arginine and lysine to various purified protein diets improved chick weights only when these amino acids were added to casein and casein-zein diets respectively. The improved weights were statistically significant when arginine was added to a casein diet, but not when lysine was added to a casein-zein diet.

The improved weight gain noted by the addition of arginine to casein diets was thought to be due to an improved amino acid balance in the blood. The arginine addition increased the free arginine of the blood only a few micrograms per milliliter and also increased lysine, proline and serine while decreasing threonine, glutamic acid, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine and ammonia. Aspartic acid and glycine remained at essentially the same concentration. Thus, the improved weight gains can be explained on the basis of an improved amino acid balance in the blood as excessive essential amino acids available in casein were not absorbed to the extent that they were on low-arginine diets, but were regulated by the addition of arginine.

There was also a significant increase in free blood plasma lysine due to dietary arginine additions to casein-zein diets. This increase in free lysine of the blood ex-

plains the increase in tyrosinase activity found with high levels of lysine and arginine, and also the moderation of depigmentation of birds on low-lysine, high-arginine diets, which have been observed in previous experiments at this laboratory.

There was a generally consistent increase in the microflora counts of coliform, lactobacilli, total aerobes, total anaerobes, staphylococci, streptococci and molds and yeast of young chicks when arginine was added to diets based on different purified protein sources. This increase in microflora population was also noted on casein and casein-zein diets with dietary lysine additions and also when lysine and arginine were added in combination.

A possible explanation for the increased microflora counts observed in these experiments, other than the increase of lysine and arginine per se, or a combination of both may be due to the influence of these dietary amino acids on the concentration of these and other amino acids absorbed into the blood. The decrease in the amounts of a majority of the amino acids absorbed into the blood when lysine or arginine was added to the diet could cause an excess of the unabsorbed portion of these amino acids in the intestinal tract. The combination of these unabsorbed amino acids may possibly be responsible for the increase in microflora found in birds on casein diets.

CONCLUSIONS

1. Increasing arginine from 1.2 to 1.8 percent improves growth and feed efficiency of chicks on casein diets.

2. Added arginine to purified diets based on zein, gelatin-casein, Drackett protein or a casein-zein combination does not improve chick growth.

3. Increasing arginine from 1.2 to 1.8 percent of the diet, when casein is the source of protein, increases the blood plasma levels of arginine, lysine, proline and serine while the levels of threonine, glutamic acid, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine and ammonia are decreased. Aspartic acid and glycine remained about the same.

4. The addition of lysine to a casein diet or to a casein-zein diet increases the free lysine of the blood plasma and decreases the blood arginine concentration.

5. The addition of arginine to a casein-zein diet increases both the free arginine and lysine in the blood plasma.

6. The addition of arginine to casein, zein, gelatin-casein, Drackett protein and casein-zein diets generally increased the coliform, lactobacilli, total aerobes, total anaerobes, staphylococci, streptococci and mold and yeast counts of the anterior portion of the intestinal tract.

7. The addition of lysine alone and in combination with arginine to casein and casein-zein diets generally in-

creases the microflora counts of the anterior portion of the intestinal tract.

8. Different purified protein sources do not influence the microflora count regardless of influence on chick growth.

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APPENDIX

Table 12. Vitamin premix

Vitamin	Quantity contributed by premix to one pound of complete ration
Vitamin A, I.U.	4,540.0
Vitamin D_3, I.C.U.	681.0
Vitamin E, I.U.	10.0
Inositol, mg	60.0
Folic acid, mg	1.5
Para-aminobenzoic acid, mg	30.0
Niacin, mg	40.0
Calcium pantothenate, mg	10.0
Riboflavin, mg	4.0
Thiamine hydrochloride, mg	2.0
Pyridoxine hydrochloride, mg	2.0
Menadione, mg	2.0
Ascorbic acid, mg	100.0
Vitamin B_{12}, mcg	10.0
Biotin, mcg	100.0

Table 13. Mineral premix

Mineral	Quantity contributed by premix to one pound of complete ration
Sodium chloride, %	0.50
Calcium, %	1.20
Phosphorous, %	0.64
Zinc, mg	2.93
Potassium, gm	5.14
Manganese, mg	32.00
Iron, mg	41.60
Cobalt, mg	1.13
Copper, mg	3.15
Magnesium, mg	400.00
Iodine, mg	1.64

Table 14. Analyses of variance of chick weight and feed efficiency of Experiment IV

Source of variation	d.f.	Casein diet 2-week chick weight M.S.	Feed efficiency M.S.
Arginine	1	2,606.5*	0.12*
Lysine	1	2,171.4*	0.11*
Arginine x lysine	1	92.2	0.07
Error	4	252.5	0.01
	7		
		4-week chick weight	Feed efficiency
Arginine	1	40,242**	1.53
Lysine	1	26,366**	2.14
Arginine x lysine	1	3,659	1.60
Error	4	666	0.34
	7		
		Casein-zein diet	
		2-week chick weight	Feed efficiency
Arginine	1	32.4	0.21*
Lysine	1	1,656.0	0.00
Arginine x lysine	1	15.4	0.09
Error	4	158.1	0.02
	7		
		4-week chick weight	Feed efficiency
Arginine	1	1,570.9	0.1100**
Lysine	1	542.9	0.0200*
Arginine x lysine	1	232.1	0.0009
Error	4	548.1	0.0025
	7		

*Significant difference at P = .05 or less.

**Significant difference at P = .01 or less.

Table 15. Analyses of variance of the free amino acids and ammonia in blood plasma of chicks in Experiment IV

Source of variation	d.f.	Casein diet: Lysine M.S.	Casein diet: Histidine M.S.
Arginine	1	244.1	359.1
Lysine	1	3,693.6*	1,082.4
Arginine x lysine	1	774.3	468.7
Error	12	633.3	516.1
	15		
		Ammonia	**Arginine**
Arginine	1	4.80	62.4
Lysine	1	5.20	5.8
Arginine x lysine	1	117.40	15.1
Error	12	19.43	33.5
	15		
		Casein-zein diet: Lysine	**Casein-zein diet: Histidine**
Arginine	1	105.1	78.7
Lysine	1	4,893.0	164.4
Arginine x lysine	1	2,964.8	74.5
Error	12	159.8	52.6
	15		
		Ammonia	**Arginine**
Arginine	1	32.8	2,978.40**
Lysine	1	34.0	463.30**
Arginine x lysine	1	5.3	164.50
Error	12	17.1	41.57
	15		

*Significant difference at P = .05 or less.

**Significant difference at P = .01 or less.

Table 16. Analyses of variance of chick weight and feed efficiency of Experiment V

Source of variation	d.f.	2-week chick weight M.S.	Feed efficiency M.S.
		Casein diet	
Arginine	1	2,801.90**	0.61
Lysine	1	508.80	0.00
Arginine x lysine	1	73.30	0.15
Error	4	267.98	0.34
	7		
		Casein-zein diet	
Arginine	1	3.00	0.0
Lysine	1	95.20	0.1
Arginine x lysine	1	18.90	0.9
Error	4	133.85	10.2
	7		

*Significant difference at P = .05 or less.

**Significant difference at P = .01 or less.